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THE PROTEIN COMPONENTS OF SUBCELLULAR STRUCTURES
INVOLVED IN PROTEIN SYNTHESIS

by

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Thesis submitted for the Degree of
Doctor of Philosophy
of the University of Glasgow

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I. INTRODUCTION

General comments on protein synthesis

The existence of proteins as characteristic and essential components of living matter has been known for over a century, but it is only recently that some insight has been gained into the mechanism by which they are synthesised. Plants and many micro-organisms can synthesise proteins from simple inorganic compounds such as ammonia or nitrate and carbon dioxide, but animals require preformed amino acids, which they generally obtain in the form of protein.

The proteins of the diet are broken down in the animal body by means of a variety of proteolytic enzymes, and at one time it was thought that these enzymes could also catalyse the reverse reaction, the recombination of amino acid residues to form peptides and proteins. The synthesis of peptide bonds by a condensation reaction was demonstrated in 1938 by Bergmann and Bruton, and proteinases were later shown (Johnston, Nycek and Bruton, 1950) to be capable also of forming peptides by a transamidation type of reaction where an amide bond already existed. Clearly, however, there must be some mechanism controlling the order in which amino acids are put together to form specific proteins, and since the formation of a peptide bond is an endergonic reaction there must be some energy source coupled to the synthetic process.

The occurrence of large amounts of ribonucleic acid (RNA) in tissues where protein was being synthesised (Caspersson, 1941; Brachet, 1942) suggested that RNA might be the factor directly controlling the organisation

of amino acid residues into various specific proteins. The RNA was visualised as a template on which amino acids were laid down, the pattern of the template being derived, presumably, from the deoxyribonucleic acid (DNA) of the cell. The observations that the incorporation of amino acids into the protein of liver slices and homogenates is inhibited by lack of oxygen or addition of dinitrophenol (DNP) (Frantz, Zamecnik, Reese and Stephenson, 1948; Siekevitz, 1952) or of cyanide (Winnick, Friedberg and Greenberg, 1947) suggested that the energy for peptide formation is derived from oxidative phosphorylation processes, and it was suggested by Lipmann (1941, 1949) that amino acids are activated by reaction of their carboxyl groups with adenosine triphosphate (ATP) to form a phosphoric acid anhydride.

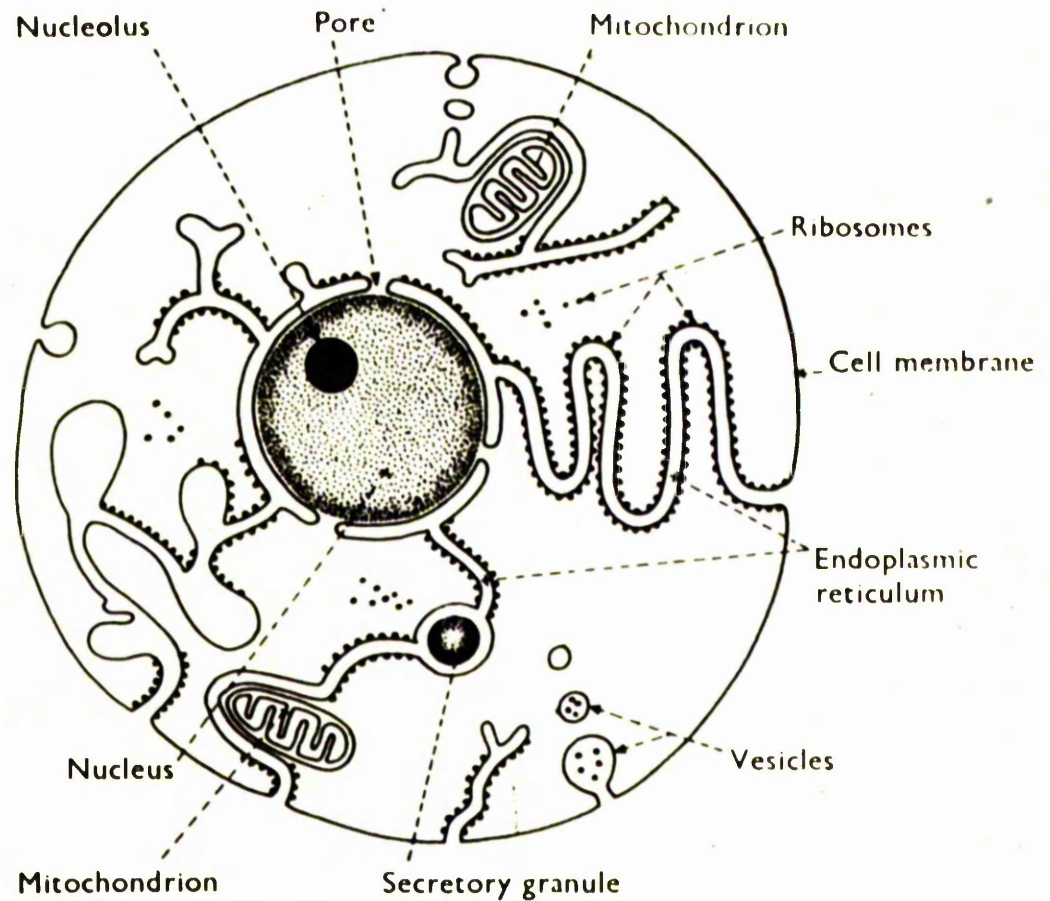
Recent advances in ultracentrifugation techniques have led to more detailed fractionation of disrupted cells (Claude, 1946; Schneider, 1948; Zamecnik and Keller, 1954), and uptake of radioactive amino acids have been demonstrated in cell-free systems (Keller, Zamecnik and Loftfield, 1954; Hoagland, Keller and Zamecnik, 1956; Hultin and Beskow, 1956). Although it is probable that the cell nucleus can carry out protein synthesis (Allfrey, Mirsky and Osawa, 1957), most proteins appear to be formed in the cytoplasm, and it is this fraction that has been most intensively studied.

Subcellular structures involved in protein synthesis

Before reviewing present theories on the role of the various structures of the cell cytoplasm in protein synthesis, we must consider briefly their cytological features and chemical nature.

A diagrammatic illustration of the components of a secretory cell is

FIGURE 1



Schematic representation of a 'typical' animal cell.

(Davidson, 1960)

given in Fig. 1, which is based on recent work by electron microscopy (Palade and Siekevitz, 1956). The largest cytoplasmic components are the mitochondria, which produce their own proteins (Reis, Coote and Work, 1959), but are probably not the main site of protein synthesis in the cell. Another prominent feature of secretory cells is the endoplasmic reticulum, which appears to consist of membranous vesicles forming a complex system extending throughout the cell, possibly continuous with the cell wall. Porter (1954) suggested that this reticulum is to be found in all cells, and Palade (1955) observed it in a large number of types of mammalian cell, although not in mature erythrocytes. Edwards et al (1956) found that it was also absent in high-frequency insect muscle, and it has been suggested that there is a difference in type of reticulum between secretory and non-secretory tissues (Birbeck and Mercer, 1960). Wakid (1960), however, has published electron micrographs of uterine muscle homogenates showing classical microsomes, and Hallinan (unpublished) has observed membranous structures in reticulocytes. Probably there is some endoplasmic reticulum in most animal cells, although its morphology may vary with the cell type. It is reduced in malignant cells in liver, or may even be almost absent in the case of rapidly growing tumours (Howatson and Ham, 1955; Novikoff, 1957). In general the reticulum is most prominent in tissues which are active in protein synthesis.

The endoplasmic reticulum consists of a network of vesicles or cisternae, which in the most commonly seen form are covered with dense granules of ribonucleoprotein material. The vesicles shrink or swell osmotically

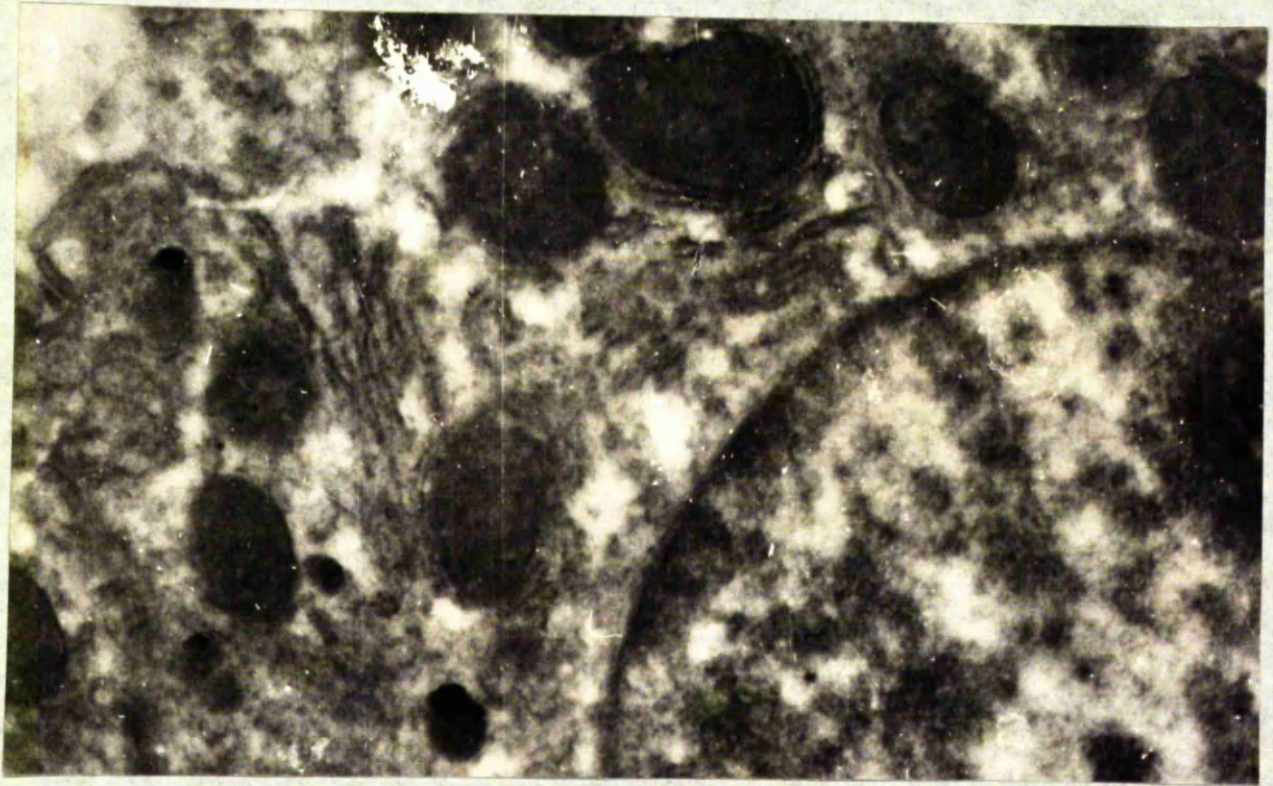
in sucrose solutions of different concentration, i.e. they are envelopes rather than solid structures. The granules attached to this type of reticulum are small (about 150-200 Å in diameter) and dense, resembling the free granules (ribosomes) seen in the acinar cells of the pancreas (Palade and Siekevitz, 1956b; Sjöstrand, 1956), although not in liver cells. Besides this granule-bearing reticulum, the rough endoplasmic reticulum, a second type consisting of membranes without granules, the smooth endoplasmic reticulum, has been observed in many tissues (Palade and Siekevitz, 1956a; Moule, Rouiller and Chauveau, 1960). The smooth reticulum occurs in liver cells, especially in the Golgi complex (Clermont, 1956), but it is much less prominent than the rough form. It seems to be associated with glycogen synthesis (Porter, 1960).

It has been suggested that the rough endoplasmic reticulum of the cytoplasm is continuous with both the Golgi complex (Palade, 1960) and the cell membrane (Palade, 1956; Epstein, 1957). If so, it might be expected to function as a transport system, e.g. for the export of material secreted by the cell. In exocrine pancreas cells small granules, chemically similar to zymogen granules (Siekevitz and Palade, 1958), have been observed within the vesicles of the reticulum (Weiss, 1953; Palade, 1956). It has been suggested (Hirsch, 1960) that such secretory products are removed after synthesis from the ribosome and carried within the reticulum to the Golgi complex, where they are condensed to form zymogen granules (Sjöstrand and Hanson, 1954) ready for secretion, presumably by passage through the reticulum to the cell membrane.

Recent work by Barer, Joseph and Meek (1960) suggests that the reticulum probably arises from the nuclear membrane, from which it appears to peel off in parallel layers. This "stacking" is characteristic of the endoplasmic reticulum of secretory cells, which tends to orientate itself in layers throughout the cell. Barer et al also noted a connection between the mitochondria and the production of endoplasmic reticulum, and found that mitochondria tended to gather round the nucleus during prophase. In the course of the work described in this thesis we have noticed that the mitochondria of the liver of fasted rats often show layers of reticulum lying beside their membrane (see Fig. 2) in a pattern resembling that seen beside the nuclear membrane. The endoplasmic reticulum is reduced in the liver of rats fasted for several days (Fawcett, 1955; Bernhard and Rouiller, 1956), and its regrowth some hours after a protein meal has been demonstrated. It was found that a meal of carbohydrate was much less effective. The regrowth appeared to take place first at the periphery of the cell and round the nucleus. In both sites it is associated with the mitochondria. The new membranes formed seem to be without granules, which appear later.

The structures shown in Fig. 1 can be obtained by differential centrifugation of a tissue homogenate prepared in sucrose solution. Workers have differed slightly in the concentration of sucrose solution they have used. They have also differed in their conditions of centrifugation, in that some have continued to prepare the "heavy microsomes" by the original method of Schneider (1948) by spinning at 18,000g, and to prepare the "light microsomes" as a separate fraction by a second spin at 105,000g.

FIGURE 2



Magnification X 24,000

Electron micrograph of liver of rat starved for 5 days, showing parallel layers of endoplasmic reticulum lying alongside mitochondria. The double membrane of the nucleus can also be seen; part of the large nucleolus is at the extreme right.

The heavy microsomal fraction consists of large vesicles with attached granules, and the light microsomes of smaller vesicles and granules. In the case of the pancreas, the light microsomal fraction also includes the free ribosomes. Other workers (e.g. Keller, Zamecnik and Loftfield, 1954; Palade and Siekevitz, 1956a, 1956b; Campbell and Greengard, 1959) have prepared the heavy and light microsomes as a single fraction. Palade and Siekevitz have shown by means of electron micrographs of the microsomal pellet that it corresponds to the endoplasmic reticulum.

Recently it has been found that prolonged centrifugation of the cell sap yields a further fraction, consisting possibly of breakdown products of the reticulum (Palade and Siekevitz, 1956a; Goldthwait, 1959; Frosser, Hird and Munro, 1961).

Owing to the complex chemical nature of the cell structures, and the ease with which many of their components are degraded during preparation, chemical analysis is not always easy or very detailed. The constituents generally estimated are nucleic acids, protein and lipids (e.g. Palade and Siekevitz, 1956a) and such analysis generally involves extraction of an acid-precipitated residue with lipid solvents. It is recognised that a variety of lipid compounds is removed by these solvents, and much work has been done on the lipid and nitrogenous material so extracted. In particular, attention has been paid to amino acid complexes.

Analysis of the different structures derived from the cell indicates that they vary greatly in chemical nature. The membranous fractions of the liver cell, the mitochondria and microsomes, appear to be rich in

material extractable by lipid solvents (Spiro and McKibbin, 1956), while the ribosomes and light microsomes consist mainly of RNA and non-extractable protein. These findings reflect the lipid character of the membrane.

A variety of methods has been used for the chemical subfractionation of the structures prepared by centrifugation, especially of the microsomal fractions. Most of the reagents act by dissolving the membranous part, leaving the granules to sediment on recentrifugation. They may be detergents, e.g. Lubrol (Cohn, 1959), or bile salts, e.g. sodium deoxycholate (Strittmatter and Ball, 1952; Littlefield *et al*, 1955; Palade and Siekevitz, 1956a). Alternatively, it may be possible to remove preferentially the granular (ribosomal) component by treating with pyrophosphate (Sachs, 1957, 1958), or by disintegrating the RNA by the use of ethylene diamine tetra-acetic acid (EDTA), which is thought to act by removing magnesium (Palade and Siekevitz, 1956a; Peters, 1959). These reagents have little effect on the membranes, which can be spun down after treatment.

Protein synthesis in the cytoplasm

Let us now consider briefly the parts played by these various structures of the cell in the synthesis of proteins by the cytoplasm.

It is now believed that the first stage of protein synthesis occurs in the cell sap, where free amino acids are activated by reaction of their carboxyl groups with ATP (Zamecnik and Keller, 1954). The process has been shown to involve a pyrophosphate exchange between ATP and pyrophosphate, since only those amino acid analogues capable of catalysing such an exchange

can be incorporated (Sharon and Lipmann, 1956). Thus the activated complex is an amino acid-adenylate. The reaction requires an amino acid-activating enzyme (Hoagland, Keller and Zamecnik, 1956), found in that fraction of the cell sap which can be precipitated at pH 5. It has been shown that there are a number of such enzymes, each specific for a certain amino acid, e.g. for tryptophan (Savie, Koningsberger and Lipmann, 1956), and probably each amino acid has its own specific activating enzyme (Hoagland et al, 1956). The amino acid-adenylate does not occur in the free state, in which it would react at random with any other amino acid it happened to meet, but remains bound to the activating enzyme until the amino acid residue is removed.

The activated amino acid is then bound by a molecule of soluble RNA (sRNA), also found in the "pH 5 fraction" of the cell sap (Hoagland et al, 1958). Again, each amino acid probably has its own specific sRNA (Holley et al, 1959), but all sRNAs contain the same terminal nucleotide triplet (-cytidylic acid-cytidylic acid-adenylic acid) at the end to which the amino acid is attached (Hecht et al, 1958). The sRNA-amino acid complex then becomes bound to the microsomes, GTP being required for this process (Hoagland et al, 1958).

It is not yet certain that sRNA is an obligatory intermediate in protein synthesis. There is some evidence for other pathways for the transfer of amino acids to microsomes, e.g. Sachs (1957) and Rendi and Campbell (1959) have obtained a protein from liver (named S-protein by Sachs) which appears to be capable of carrying out such a transfer without

sRNA. Beljanski and Ochoa (1958a, 1958b) have observed a similar enzyme-catalysed pathway in Alcaligenes faecalis.

In all these systems, the actual combining of the amino acid residues seems to take place in the microsomes. It is at this stage that the arrangement of amino acids to form specific proteins occurs, and it is generally assumed that the template determining the sequence of amino acids is a high molecular weight RNA. This was at one time thought to be the ribosomal RNA, but recently it has been suggested that the template on which the amino acids are laid down is a "messenger RNA" (Cros et al, 1961), produced by the nucleus, which becomes attached to the ribosomes. Such a mechanism would enable a single species of ribosomal RNA to synthesise a variety of proteins.

Attempts have been made to discover how the four bases of RNA can provide a coding system for some twenty amino acids, and recently evidence has been put forward (Crick et al, 1961) suggesting that there may be one or more nucleotide triplets specific for each amino acid, e.g. a trinucleotide of uridylic acid may possibly be the code for phenylalanine (Nirenberg and Matthaei, 1961).

The "messenger RNA", produced in the nucleus and presumably deriving its code from the nuclear DNA, is thought to act by attaching itself to the ribosomal RNA. It has been assumed that it is in the ribosomes (the ribonucleoprotein granules of the microsomes) that protein synthesis occurs, and incorporation of radioactive amino acids has been shown to take place in this fraction (Korner, 1959). Siekevitz and Palade, too, have shown

that, in vivo, the most highly labelled fraction of chymotrypsinogen in the pancreas after injection of ^{14}C -valine is that attached to the ribosomes. It has also been shown, however, by Keller and Cohen (1961), that certain methods of isolating the ribosomes can bring about their contamination by added chymotrypsinogen, and it is possible that in Siekevitz and Palade's experiments labelled material may have been transferred to the ribosomes from damaged membranes. Korner (1961) found that although the ribosomes of rat liver ultimately reached a higher level of activity than the microsomes, the microsomal activity, relative to RNA content, increased more rapidly during the first thirty minutes of incubation with radioactive amino acids. Other evidence for a role in protein synthesis for the microsomal membrane comes from studies by von der Decken, Löw and Hultin (1960) on the effect of phalloidin on guinea pig liver microsomes. They found that, although the incorporation of amino acids was inhibited by phalloidin, the ribosomes prepared from the microsomes by detergent treatment retained their full ability to incorporate amino acids, at least during the early stages of liver damage. It thus appears that the ability of the microsomes to incorporate amino acids depends partly on the membranous component.

In a series of experiments on the synthesis of a specific protein (serum albumin) by ribonucleoprotein particles, Campbell (1960) has so far failed to detect any serum albumin in ribosomes prepared by deoxycholate treatment of rat liver microsomes. He has also failed to demonstrate net synthesis by such particles of serum albumin. This may be because

the cell sap which is also required for the incorporation of labelled amino acids already contains large quantities of inactive serum albumin, which dilutes the activity of the newly synthesised albumin. In the intact cell, the newly formed albumin would probably be retained by the microsomal membrane.

In another series of experiments, Campbell, Greengard and Kernot (1960) have separated two fractions of albumin from liver microsomes by ultrasonic disintegration. Both in vivo, three minutes after the injection of a mixture of radioactive amino acids, and in vitro after incubation of isolated microsomes with ^{14}C -leucine, the fraction solubilised by ultrasonic treatment was much less active than the smaller, sedimentable fraction, which could be solubilised by deoxycholate and was therefore probably connected with the membrane. Peters (1959) has also obtained evidence that newly synthesised albumin is bound to the lipid part of the liver microsomes, and can be released by treatment with deoxycholate. He finds that, although the albumin seems to be formed within two or three minutes, it is not released from the cell for about twenty minutes. Such a long interval, he thinks, suggests that the process involved is not purely chemical, but may involve passage through a membrane. Campbell, Greengard and Kernot (1960) also found that newly synthesised albumin was not released from rat liver microsomes, and suggested that the membrane retains the albumin until it reaches the cell wall. Possibly other factors are involved in the release of newly synthesised proteins from the membranes, since Grabowski and Munro (1960) found that amylase was released from

pancreatic microsomes only when the incubation medium contained free amino acids. The process appeared to be one of amylase release by the amino acid, rather than of further protein synthesis, since a single amino acid sufficed, and there was no increase in the total amount of amylase in the preparation during the release of the bound fraction. Certainly, if the endoplasmic reticulum is continuous with the cell membrane, it might well act as a channel for secreted proteins, possibly carrying them first to the Golgi complex for condensation, as in the case of the zymogen granules of the pancreas. It is curious that, although the intracisternal granules which are thought to be the precursors of these zymogen granules are quite conspicuous in pancreas cells, no accumulation of material appears to have been observed within the vesicles of the liver cell (Porter, 1960). Possibly the synthesis of albumin is a slower and more continuous process than the production of pancreatic enzymes, which occurs mainly in response to a meal.

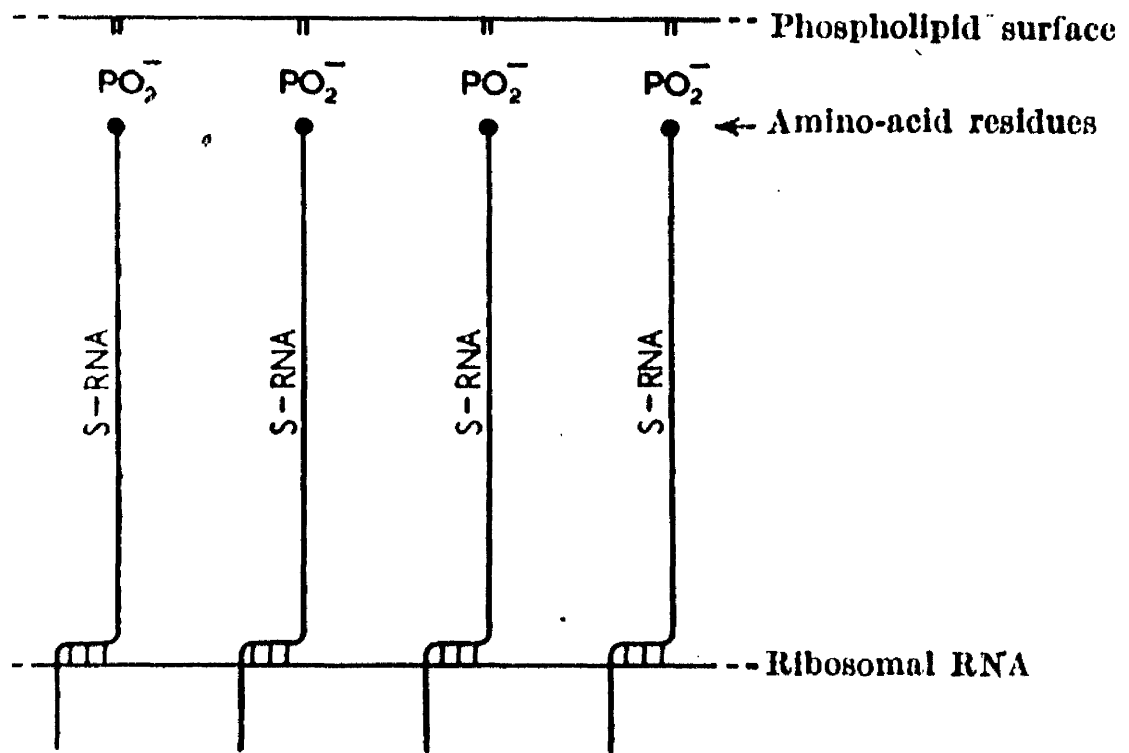
At any rate, there is a good deal of evidence to suggest that the membranous part of the microsomes plays a part in the production of proteins, whether in the actual synthesis or in the removal and transport of the newly synthesised protein. Campbell (1960) suggests that it may be involved in both processes, and that his "results are consistent with the idea that in the liver the initial incorporation of amino acids is into the RNP particles and that an albumin precursor then moves to the lipid membranes where there is a further addition of amino acids to complete the albumin molecule. The albumin then moves to the internal medium of

the vesicles where it is securely retained until it is passed through the cell membrane." If the albumin molecule is actually synthesised in part on the lipid membranes, then either those membranes must be capable of controlling the order of the amino acids added there, or the amino acid order must be determined by the ribosomes. Ribosomal control could be achieved either by the maintenance of a link between the albumin and the ribosomes, or by a system whereby the amino acids are lined up ready for attachment before the partially synthesised molecule is transferred to the membrane. If the order is controlled by the membrane itself, the controlling agent may be the RNA which has been reported within the membrane, where it forms about 20% of the total microsomal RNA (Kuff, Hogebom and Dalton, 1956; Moule et al, 1960).

Hunter and Godson (1961) have proposed a different mechanism (see Fig. 3) for binding between amino acid residues and a lipid material in the membranes. They suggest that the sRNA-amino acid complexes might be attached by their free ends to the RNA of the ribosomal particles with the projecting amino acids held at the same distance from the ribosomal RNA, which acts as a template. These amino acid residues might be aligned along the surface of a phospholipid chain, probably a phospholipoprotein, where they might form lipo-amino acid complexes. The adjacent phosphate groups might then catalyse the condensation of such complexes into a phospholipoprotein.

The membranes, as we have seen, are distinguished among the cell fraction by their high lipid content, and if they are involved in protein synthesis

FIGURE 3



Outline of structure of proposed 'template' for protein biosynthesis

(Hunter and Godson, 1961)

their lipid components may well play a part. Let us therefore consider what is now known of the uptake of amino acids by lipid substances.

Recent work on amino acid incorporation into lipid substances

A variety of substances which appear to be capable of incorporating or binding amino acids has been found in organic solvent extracts of different tissues.

1) Lipo-amino acid complexes:

Hendler (1959, 1961) examined an ethanol/ether extract of trichloroacetic acid (TCA)-precipitated material from hen oviduct. He found in it a non-polar substance which contains radioactivity after incubation of the tissue mince with labelled amino acids. The substance, which is also soluble in chloroform or acetone, is derived from the cell particles rather than the supernatant, and shows its greatest activity when the tissue has been engaged in active protein synthesis. Homogenisation of the tissue or the addition of DNA before incubation prevents the labelling of this "lipid" material. On the other hand, if the tissue is incubated before homogenisation, then on further incubation of the homogenate the proteins become radioactive, while the activity of the "lipid" fraction declines. Thus Hendler's work suggests that a non-polar, lipid-like material from cell particles is capable of incorporating amino acids in the intact cell in conditions which allow of protein synthesis, and that the resulting complex may act as a precursor of protein.

Hunter and Goodsall (1961) have isolated a lipo-amino acid complex from protoplasts obtained by lysis of Bacillus Megaterium, which shows

properties rather like those of Hendler's fraction. It is soluble in methanol or acetone, and rapidly becomes labelled when protoplasts are incubated with radioactive amino acids. This labelling does not take place when chloramphenicol is added to the incubation mixture, although the protoplasts are still capable of accumulating amino acids. Like Hendler's material, this lipid complex can act as a protein precursor when incubated with protoplasts, and the conversion has been shown to be direct rather than via the free amino acids. Its chemical nature is not certain. Although not one of the usual phospholipids, it may possibly be a phosphatidic acid. More detailed analysis of lysed protoplasts (Godson, Hunter and Butler, 1961) indicates that the earliest incorporation of ^{14}C occurs in a phospholipoprotein complex which has been isolated after centrifugation as the upper layer of the ribosome-free membrane complex. The activity declines as incubation of pre-labelled protoplasts proceeds, while that of the non-membranous protein increases. The ribosomal protein, although also active, does not show any fall with time, and Godson et al consider that it is probably not a primary source of material for protein synthesis. They suggest that the findings of workers who believe that it is, e.g. Siekevitz and Palade (1960), are perhaps due to damage by their isolation procedure to the membrane structure of the cell, with resultant transfer of labelled material to the ribosomes. Such transfer cannot be ruled out in view of the findings of Keller and Cohen with chymotrypsinogen, already mentioned.

Further evidence for the participation of a lipid material in protein

synthesis comes from the work of Gaby et al (Gaby, Golin, and Zajac, 1960; Friedmann and Gaby, 1960) on rabbit-liver, Ehrlich ascites and lymph-node cells. They have demonstrated rapid uptake of various amino acids into a lipid complex, and slower labelling of other fractions. These workers consider that the amino acids are bound to phospholipids, from which they were unable to separate them by electrophoresis. After acid hydrolysis of the complex, they were able to demonstrate the presence of free amino acids in the aqueous layer by means of paper chromatography.

Haining, Fukui and Axelrod (1960), on the other hand, have obtained from rat liver a lipid fraction which also incorporated radioactive amino acids but did not contain phosphorus. Labelling took place at a constant rate throughout the two hours of incubation, without dilution by added non-active amino acid. Different amino acids were incorporated at different rates, which were unrelated to the rate of their incorporation into protein. The cell sap, when incubated separately, gave a more active lipid fraction than the microsomal preparation incubated without cell sap, but both fractions yielded lipid fractions of lower activities when they were incubated together. No energy source appeared to be necessary, a fact which might suggest that the uptake of amino acids in this case is a purely physical adsorption, although we must bear in mind Hendler's suggestion (1961) that lipid media may contain some kind of energy source different from those, such as ATP, found in aqueous media. Fukui and Axelrod have also obtained a stable compound, probably a fatty acid acyl-amino acid, on incubating amino acids with a monoglyceride and a rat liver

acetone powder.

These findings are difficult to reconcile with the findings of other workers. One reason may be that most of Haining et al's data were obtained by the use of phenylalanine which, as both Hendler (1961) and Hunter and Goodsall (1961) have shown, behaves atypically in such incorporation systems. Wren (1960) has pointed out the danger that lipo-amino acid artefacts may be formed as the result of binding of free amino acids in vitro by lipids in the presence of proteins and organic solvents. Binding of labelled amino acids might thus take place while the tissue was being extracted with lipid solvents. In the case of Haining et al's experiments, however, it is unlikely that any free amino acids remained by the time the residue was exposed to organic solvents.

2) Phosphatidopeptides:

A number of phosphatidopeptides have also been isolated from various tissues, and there is some evidence that they may play a part either in the synthesis of protein or in its transport through lipid membranes. Thus Hokin and Hokin (1958, 1959) found that the turnover of phosphorus in phosphatidic acid of brain is increased by acetyl choline, the effect being chiefly in the microsomal fraction, and being dependent on the structural integrity of the microsomal fragments. An increased turnover of phosphatidic acid is also observed in pancreas and pituitary tissue, associated with secretory activity. Hokin suggests that phosphatidic acid is involved in a mechanism for the transport of polar secretory material across a lipid membrane. A similar effect has been observed in chicken

pancreas slices by Huggins (1930), who obtained stimulation by acetylcholine both of amylase secretion and of phosphorus turnover in a phosphatidopeptide and in phospholipid. Huggins and Cohn (1959) have obtained from many mammalian tissues a phosphatidopeptide which is soluble in chloroform/methanol/HCl (200:100:1 by volume), but not in warm ethanol/ether, and which is therefore not identical with Hendler's non-polar lipid. It is thought to be similar to a phosphatidopeptide isolated from brain tissue by Folch (1952), although probably not identical with it, and has been found in many tissues. Huggins and Cohn (1959) suggest that the amount of phosphatidopeptide phosphorus may vary with the nutritional state and health of the animal, an interesting point in view of the known variation of the amount of the endoplasmic reticulum with diet (Lawcett, 1955; Bernhard and Rouiller, 1956). The distribution within the cell, however, at least in the kidney cortex, does not support any attempt to relate the phosphatidopeptide to the endoplasmic reticulum, since it is very poor in the microsomes and richest in the mitochondria. This finding may, however, be connected with the fact that kidney cortex is not a secretory tissue.

Both Folch and Huggins have succeeded in identifying a large number of amino acids in their phosphatidopeptides (fifteen and twelve respectively), but their investigations have been directed mainly towards the phosphorus component. Barnabei and Ferrari (1961) have studied phosphatidopeptide activity in incorporating amino acids, which they find to be quite high. The acetone- and alcohol/ether-soluble fraction from TOA-precipitated rat

liver also incorporates amino acids, but not to such an extent. With the exception of ethionine, which powerfully inhibits uptake into both proteins and phosphatidopeptides, factors which inhibit incorporation into protein, such as chloramphenicol, cyanide and anaerobiosis, have a much smaller effect on the incorporation into phosphatidopeptide. The authors suggest that this may be because the maximum level of incorporation into phosphatidopeptide is reached more rapidly.

It is clear, therefore, that many lipid-like substances containing amino acids have been extracted from a variety of tissues and micro-organisms, and have been shown to be capable of incorporating amino acids. These preparations may be regarded as falling roughly into two main classes. Those in the first group, such as the phosphatidopeptides of Folch and of Huggins and Gohn, have been shown to contain peptide chains. The substances in the second group are described simply as "lipo-amino acid complexes" (e.g. Hunter's B. Megaterium protoplast material) or as "amino acid-lipid complexes" (e.g. Hendler's hen oviduct material). These may also contain peptide chains rather than single amino acid residues; the possibility has not been rigorously explored, however, although Godson, Hunter and Butler (1961) did find that the most highly labelled fraction of their preparation was a phospholipoprotein.

If these two groups of complexes are related to each other, the possible relationships are (1) that they are chemically identical, (2) that the "phosphatidopeptide" substances act as precursors of the "lipo-amino acid complex" group or (3) that the "lipo-amino acid complexes" act as precursors

of the "phosphatidopeptides". The differences in solubility in various solvents already mentioned make it appear unlikely that the two types of complex are identical, but there are a number of metabolic differences also. Thus the phosphatidopeptide studied by Barnabei and Ferrari (1961) can incorporate amino acids in the presence of such inhibitors as cyanide or chloramphenicol, or in anaerobic conditions. It therefore seems probable that it is not the same substance as Hunter's lipo-amino acid complex, which is sensitive to chloramphenicol, or as Hendler's amino-acid lipid complex, which incorporates amino acids only when protein synthesis is taking place and would presumably be inhibited by cyanide. For the same reason, the phosphatidopeptide cannot be derived from such complexes. The third possible relationship between these two types of complex is that the phosphatidopeptide may be a precursor of the "lipo-amino acid complex". This is acceptable only if the latter contains a peptide chain at least as long as that of the phosphatidopeptide; there is no information on this point.

Alternatively, it is possible that there is no direct relationship among the various lipid complexes which have been obtained by different workers. They may instead form part of different routes of protein synthesis. If so, it is interesting to speculate whether they may be connected with other possible mechanisms of attachment of amino acids to the microsomes in which sRNA is not required, such as the "S protein" system of Sachs (1957) and of Rendi and Campbell (1959), or the enzyme-catalysed pathway observed in Alcaligenes faecalis by Beljanski and Ochoa (1958).

Finally, it should be noted that the measurement of amino acid uptake in a "protein precipitate" obtained by the use of TCA, or some other precipitant, may not be a valid indication of the uptake into the protein normally produced by the cell. It has been shown that lymph-node slices can incorporate radioactive glycine into a specific antibody protein, but that if the cells are mechanically damaged they no longer synthesise this specific protein (Askonas, Simkin and Work, 1957). They continue, however, to produce a labelled "protein precipitate". Hence amino acid incorporation ought, if possible, to be studied in specific types of protein which can be characterised, immunologically or by their enzymic activity, and not simply in an unidentified "protein precipitate". It is generally more convenient to study secretory proteins, such as serum albumin in the liver, or amylase or chymotrypsinogen in the pancreas, which are both specific and produced in easily detectable amounts. Most of the work on protein synthesis has therefore been carried out on secretory cells. It seems quite probable that the mechanism involved in the production of proteins to be retained in the cell may be different, and insofar as the function of the endoplasmic reticulum in the secretory cell is to provide a transport system to carry proteins to the cell wall for export, it will not be needed in a non-secretory cell. There is indeed evidence that in non-secretory cells it is scanty, and may not function even in the synthesis of proteins. The peptide chain of haemoglobin appears to be formed in the ribosomes of rabbit reticulocytes (Bishop, Leay and Schweet, 1960) and not in a membran

Summary of the present status of cell membranes and lipid materials
in protein synthesis

1. Most proteins are synthesised in the cytoplasm of the cell, and synthesis can be demonstrated in cell-free systems. It has been shown that the particulate fractions of the cell are involved.

The nature of the microsomes and ribosomes:

2. The microsomal fraction of liver contains a large amount of membranous material, the endoplasmic reticulum, consisting of vesicles covered with dense granules, the ribosomes. The endoplasmic reticulum probably arises from the nuclear membrane and appears to form a continuous system throughout the cell, probably connected with the cell wall.
3. The membranes and granules can be separated by chemical means. The membranes are rich in phospholipid material, while the granules consist mainly of RNA and protein.

The role of the cell sap and of the microsomes in protein synthesis:

4. The chief mode of incorporation of free amino acids involves activation by ATP and enzymes in the cell sap, binding by a low molecular weight soluble RNA of the cell sap, and subsequent transfer to the microsomes. There is probably a specific activating enzyme and sRNA for each amino acid.
5. The amino acid residues are condensed in the microsomes to form proteins. The specificity of a protein is determined by high molecular weight RNA, either part of the ribosomes or, perhaps, a "messenger RNA" attached to the ribosomal RNA but derived from the nucleus.
6. Serum albumin appears to be synthesised in association with the

membranous part of liver microsomes, but it is not released immediately (Peters, Campbell). Possibly the endoplasmic reticulum acts as a transport system for the export of such secretory proteins. The microsomal membrane may play an important part in the synthesis of secretory proteins (Campbell, Peters), possibly by providing a surface along which the sRNA-bound amino acid residues align themselves (Hunter) before condensing to form a protein or phospholipoprotein. The mechanism of synthesis of non-secretory proteins may, however, be different.

Amino acid incorporation by lipid substances:

1) Amino acid-lipid complexes:

7. An acetone- or methanol-soluble "lipo-amino acid complex" from protoplasts of B. Megaterium (Hunter), and a non-polar substance soluble in ethanol/ether after TCA precipitation of hen oviduct cell particles (Hendler), have been shown to become rapidly labelled during incubation of the protoplasts or tissue mince with radioactive amino acids in conditions where protein synthesis occurs. Where protein synthesis does not occur, the "lipid" material does not become labelled.

8. The lipo-amino acid complex of the protoplasts of B. Megaterium appears to be a precursor of non-membranous protein in the bacterium, whereas the ribonucleoprotein of the protoplasts is not. Godson and Hunter suggest that possible transfer to ribosomes of labelled material from membranes damaged during the isolation procedures may account for findings by Siekevitz and Palade (1960) which are inconsistent with this picture.

9. Rapid incorporation of labelled amino acids into lipid fractions has also been found in Ehrlich ascites, rabbit-liver, and lymph-node cells (Gaby). The amino acids are firmly bound to phospholipid-like fractions.

2) Phosphatidopeptides:

10. A role has been suggested for phosphatidic acid in the transport of polar secretory materials across lipid membranes (Hokin), and phosphatido-peptides have been isolated from many tissues (Huggins). Their abundance in the mitochondria, which are enclosed in a membrane, supports the idea of their involvement in such a transport mechanism, but the microsomes contain very little phosphatidopeptide, at least in kidney cortex.

11. The phosphatidopeptides also incorporate amino acids rapidly, but are little affected by inhibitors of protein synthesis. They are thus neither identical with nor derived from the lipo-amino acid complexes described in para. 6. Although they may be precursors of those fractions, it is also possible that they may represent part of a different process.

12. There are thus several different systems containing lipo-amino complexes or lipo-peptides for which a metabolic role in protein synthesis or secretion has been suggested.

Purpose of the present Experiments

It is clear from the foregoing description of nitrogenous substances extractable by organic solvents that they may well play a part in protein synthesis, but it is evident also that as a class of compounds they lack both chemical and morphological definition. We have, therefore, undertaken an examination of their occurrence in the liver cell, and related it to cell structure, to the nutritional state of the animal and to incorporation of labelled amino acids.

The initial studies were carried out in conjunction with an examination of the claim of Venkataraman and Lowe (1959) and of Venkataraman (1960) that up to thirty per cent. of the cytoplasmic NA might be removed by treatment of TCA-precipitated liver material with 95 per cent. alcohol. In the course of investigations of this claim we observed that a large amount of nitrogenous material other than phospholipid was being removed by such treatment. It is known that serum albumin is dissolved by acid alcohol (Levine, 1954; Debro, Tarver and Korner, 1957), but the losses we observed were too great to be accounted for on the basis of present estimates (Peters, 1959) of the albumin content of liver. Experiments were, therefore, carried out to discover the effect of prolonged exposure to alcohol after TCA precipitation, and the comparative efficiency of different solvents in removing nitrogen.

The chemical nature of the extracted material was studied by means of protein and amino acid reactions, paper chromatography, electrophoresis, protein and lipid stains, and washing with water by the procedure of Folch,

Lees and Sloane-Stanley (1957).

The distribution of the material, which may conveniently be referred to as "lipid N" (a term which does not include the phospholipid N), was studied in the various fractions of the cytoplasm of the liver cell. The results of this analysis pointed to a possible connection with the endoplasmic reticulum, so attention was paid especially to the microsomes. Microsomal pellets, prepared by ultracentrifugation, were treated with a variety of reagents - pyrophosphate, deoxycholate, lysolecithin and Staph. aureus haemolysin - in order to separate the granules from the membrane. The "lipid N" content of each fraction was then analysed.

In another series of experiments designed to establish a possible relationship with the endoplasmic reticulum, the "lipid N" content of the cytoplasmic fractions, and especially of the microsomes, was measured in animals in different dietary states. Attempts were made to correlate chemical differences with the appearance of the intact cell or of the microsomal pellet, as revealed by the electron microscope.

The metabolic behaviour of the "lipid N" was studied in vivo by the intravenous injection of ^{14}C -leucine into rats in different dietary states. The uptake of the radioactivity into the various chemical and morphological fractions of the liver was measured at various times after the injection.

EXPERIMENTAL

Animals:

Female rats were used in one experiment, but otherwise the animals were male albino rats or albino mice from the departmental colony. The rats weighed 160-180 g. or 180-200 g. in any one experiment, and the mice about 30 g.

Feeding:

The animals were maintained on a commercial stock diet (86) with water ad lib., or on special diets based on Tables 1 - 2.

In the experiments involving special diets, the animals were kept in individual cages, and trained to take two meals a day, at about 9.30 a.m. and 3 p.m. The morning feed consisted of 1 g. of vitamin/mineral/roughage mixture with the appropriate supplement of carbohydrate or fat to provide energy. The afternoon feed consisted of 4.2 g. of "high protein diet" or "low protein diet". The feeds were weighed out into glass ointment jars, and moistened with water. The rats soon learned to consume each meal within a short time of receiving it.

As a rule, to ensure that they were in the post-absorptive state, the animals were fasted for 18 hours before being killed. "Protein-fed" rats, after fasting for 18 hours, were given 2.5 g. of casein at the appropriate time before the experiment. The casein was moistened with water, and, since the powdery casein was unpalatable, a trace of sodium bicarbonate was added to dissolve it slightly.

In this way, it was possible to perform experiments on rats in

TABLE 1

A. Basic Diet

<u>High protein diet</u>		<u>Low protein diet</u>	
Margarine	42 g.	Margarine	42 g.
Potato starch	69 g.	Potato starch	189 g.
Glucose	69 g.	Glucose	189 g.
Casein	<u>240</u> g.		420 g.
	420 g.		

B. Vitamin/mineral/roughage mixture

Sodium chloride	32.5 g.	
Salt mixture "446"	130 g.)	} see Table 2.
Vitamins in starch	250 g.)	
Agar powder	62.5 g.	
Vitaminised margarine	77.5 g.	

C. Supplements

Normal carbohydrate supplement:

5 g. of a mixture of starch and glucose (dextrose monohydrate) (5:1 w/w)

Glucose supplement:

3.8 g. glucose monohydrate (Dextrosol)

Fat supplement:

1.65 ml. olive oil (BP) (the calorific equivalent of 3.8 g. glucose)

TABLE 2

Mixtures used in making up vitamin/mineral/roughage mixture

<u>Salt mixture "446"</u>		<u>Vitamins in starch</u>	
Na Cl	24.3 g.	Pyridoxine HCl	25 mg.
K citrate	533 g.	Riboflavin	25 mg.
K ₂ HPO ₄	174 g.	Thiamine HCl	25 mg.
CaPO ₄ ·H ₂ O or Ca·H ₄ (PO ₄) ₂)	800 g.	Nicotinic acid	100 mg.
CaCO ₃	368 g.	Menaphthene (Vit. K)	5 mg.
Fe ⁺⁺⁺ citrate·3H ₂ O	36 g.	Biotin	5 mg.
CuSO ₄ ·5H ₂ O	0.4 g.	Ca pantothenate	0.2 mg.
CaCl ₂ ·6H ₂ O	0.2 g.	p-amino benzoic acid	0.5 mg.
K ₂ Al ₂ (SO ₄) ₂ ·24H ₂ O	0.2 g.	Inositol	1.0 g.
NaF	0.002 g.	Choline Cl ⁷	10 g.
MgCO ₃	92.0 g.	Folic acid	trace
MnSO ₄	2.8 g.	Potato starch	to 100 g.
KI	0.1 g.		
ZnCO ₃	0.1 g.		
	2250 g.		

precisely defined dietary states e.g. after a known period of fasting or at a given time after a protein meal. A variety of dietary groups could be produced, and those used in our experiments were as follows:

- (a) Animals fasted for 18 hours after maintenance on a diet providing no protein and either a low energy intake (i.e. with no supplement) or an adequate energy intake from added carbohydrate or fat.
- (b) Animals fasted for 18 hours after maintenance on diets containing adequate protein fed at different energy levels, as in (a).
- (c) Animals prepared in the same way as group (b), but absorbing amino acids from a protein meal consumed 1 - 6 hours beforehand.

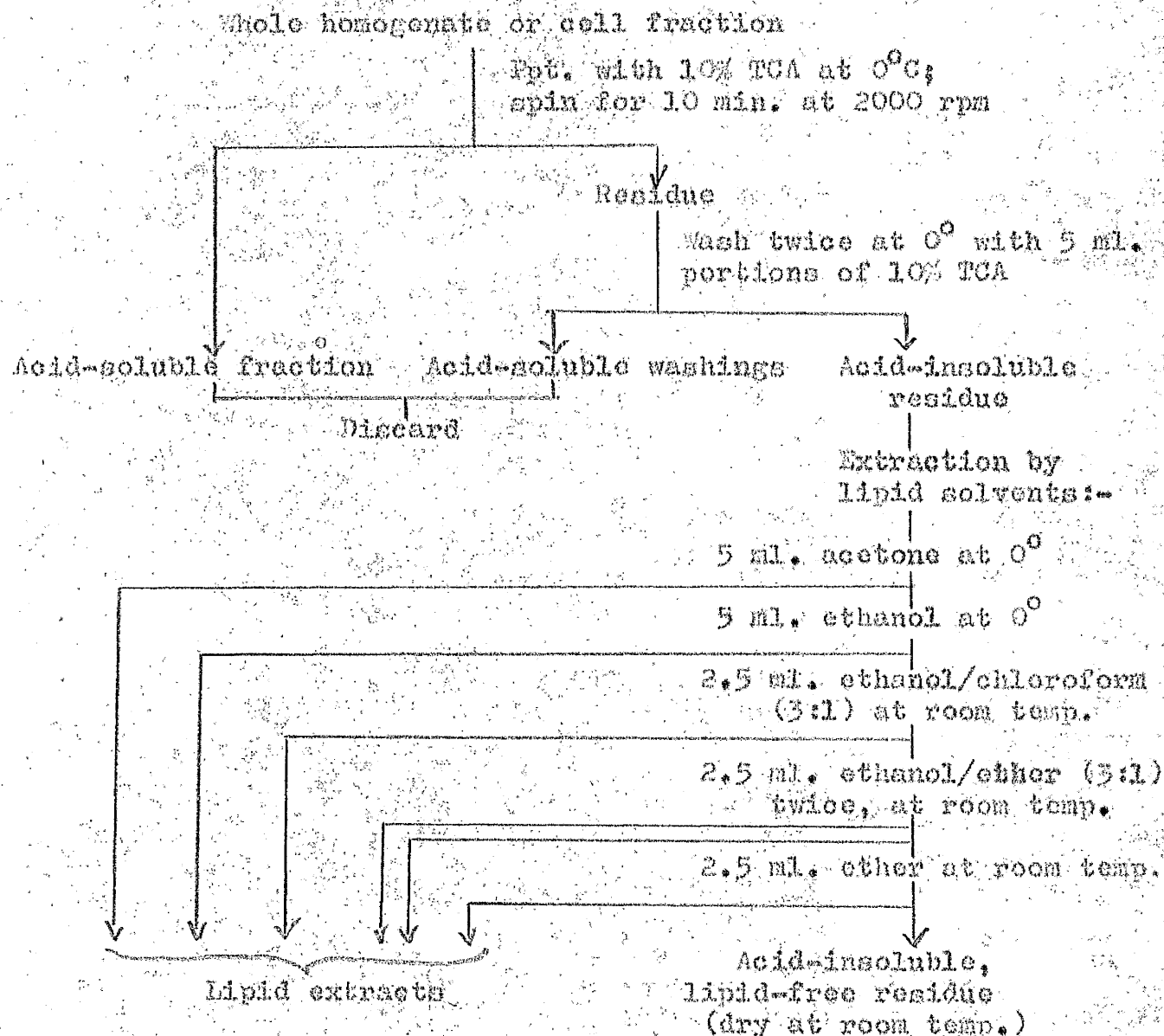
Tissue fractionation:

Tissues were prepared for analysis either as whole homogenates or as cell fractions.

A. Whole homogenates. The animals were killed by stunning. The liver was removed as quickly as possible, freed of any obvious fibrous material and blotted free of blood. It was immediately chilled in ice, weighed and homogenised in ice-cold distilled water (usually 9 volumes) by means of a Waring blender. Portions of 5 ml. of homogenate were immediately mixed with 2.5 ml. of ice-cold 30% TCA and kept in ice for 10 minutes (see Fig. 4). The precipitated protein and nucleic acids were centrifuged down at 2000 r.p.m. for 10 minutes at 0°C, and the precipitate was washed twice with 5 ml. cold 10% TCA, all washings being discarded. It has been found that three such extractions with 10% TCA are adequate for the removal of acid-soluble P compounds (Moulé, 1953) and acid-soluble glycogen

FIGURE 4

Analytical procedure



(Stetten, Katzen and Stetten, 1958).

Where appropriate, the precipitate was then extracted successively with the lipid solvents shown in Fig. 4, the residue being carefully stirred with a glass rod during each extraction. The solvents used after acetone are those recommended by Hutchison and Munro (1961), who discuss the rationale of the extraction procedure with particular reference to phospholipid solubilities. The use of acetone will be discussed in the "Results" section; it may be noted here that acetone removes most of the water and residual TCA before the residue is exposed to ethanol. While some TCA may remain in the precipitate even after two or three extractions with organic solvents (Marko and Butler, 1951), degradation of DNA to apurinic acid on treatment with ethanol/ether in the presence of this TCA is unlikely to occur unless the extraction is carried out with hot solvent. In our experiments the residue was extracted at room temperature. The insoluble material was spun down and finally allowed to dry at room temperature. Unless otherwise stated, the lipid extracts were pooled. When no lipid solvents were used, the acid-insoluble residue was drained, stirred up with 1 ml. of ice-cold water and neutralised by careful addition of 2N NaOH. The suspension was kept at 0° until the lipid-extracted series had dried, so that both series could be worked up together.

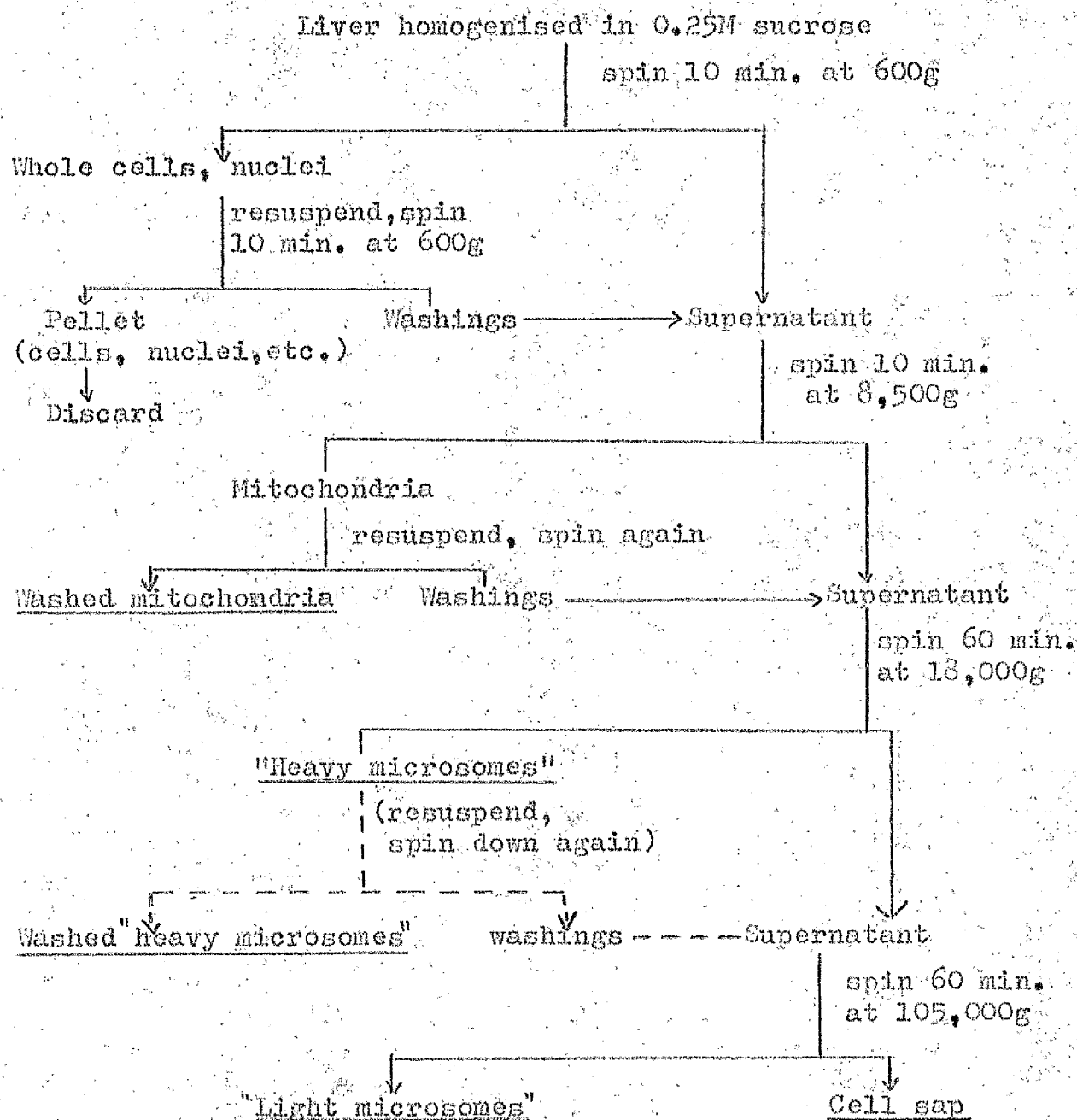
B. Cell fractions. The scheme for fractionation in 0.25 M sucrose was that of Schneider (1948), which provides the nuclear, mitochondrial and heavier microsomal particles. More recently, the introduction of centrifugation at 105,000 g for 1 hour has allowed the separation of a

further fraction, the "light microsomes". Many authors in fact combine the two microsomal fractions (e.g. Palade and Siekevitz, 1966a) but we have found some chemical differences and continue to subdivide them.

The liver was removed as quickly as possible, rinsed in ice-cold sucrose solution, blotted and weighed, and was then homogenised in 0.25 M sucrose solution at 0°C in a Potter homogeniser with a plastic pestle for about 15 seconds at high speed. The volume was made up to 50 ml. with sucrose solution, and portions of 2.5 ml. (equivalent to 250-300 mg. of liver) were transferred to 10 ml. plastic tubes and fractionated as shown in Fig. 5. The procedure was, as far as possible, carried out for quantitative recovery, but, in order to obtain a relatively pure microsomal fraction, the "fluffy" layer of the mitochondria was included in the mitochondrial fraction even though it no doubt contained some large microsomes. As shown in the scheme, the mitochondrial and (sometimes) the heavy microsomal pellets were resuspended in sucrose solution and spun down again, the washings being added to the supernatant from the first spin. The light microsomes were not washed because of the longer time required, but in any case washing was desirable chiefly where small structures might have been trapped and spun down with larger particles, and was therefore probably less necessary in the case of this, the last, particulate fraction. The mitochondria, heavy and light microsomes and cell sap were taken for analysis, and were usually frozen for storage as soon as prepared, if several fractions were to be analysed. Later (usually the next day) they were allowed to thaw at 0°C, and treated with 10% TCA (or 0.5 vols. 30% TCA in the case of the cell sap) to precipitate with 10% TCA (or 0.5 vols. 30% TCA in the case of the cell sap) to precipitate

FIGURE 5

Cell fractionation



(All carried out at 0°C)

proteins and nucleic acids. The precipitate was washed with 10% TCA, and either extracted with lipid solvents or simply drained, neutralised and kept at 0° for about 2 hours as described for the whole homogenate.

Analysis:

The end-products of these procedures were analysed either by the Schmidt and Thannhauser (1945) method or by a modification of the POA extraction method of Schneider (1945) as follows:

1. Schmidt-Thannhauser separation:

The dried powder or drained residue was incubated for 16 hours at 37° with 1N NaOH (2 ml. for whole homogenates, 1 ml. for cell fractions). It has since been found (Fleck and Munro, in press) that a much shorter incubation period (e.g. 1 hour) is adequate for the digestion of RNA to acid-soluble fragments. The harmful effects of longer incubation, however, such as degradation of protein and possible deamination of cytidylic acid, would not affect the values for RNA obtained by the orcinol reaction. RNA was measured by ultraviolet absorption only when the period of digestion with alkali had been reduced to 1 hour, so that protein interference was not serious.

If separation of DNA and RNA was required, the alkaline digest was then chilled in ice, neutralised with 1 ml. HCl (2N for homogenates, 1N for cell fractions), and the DNA and undigested protein precipitated by the addition of 0.5 vols. cold 30% TCA, i.e. enough to give a final concentration of 10%. The precipitate was washed twice with 2 - 3 ml. cold 5% or 10% TCA and the washings were added to the original supernatant.

The supernatant fraction was then made up to 10 ml. with distilled water. The residue was dissolved in 1 ml. 1N NaOH (or 2 ml. if necessary) at room temperature and made up to 10 ml. with distilled water. If separation of RNA and DNA was not required, analysis was carried out directly on the whole alkaline digest (diluted to 10 ml. with water).

2. ICA extraction:

In routine analysis, the dry powder, or neutralised residue, was extracted with frequent stirring for 20 minutes at 70° with 5 ml. 1.0N ICA, and then with a further 4 ml. The extracts, containing the RNA, were pooled and made up to 10 ml. with water. The residues, dissolved in water + 2 ml. 2N NaOH, were also made up to 10 ml. These conditions were found to be optimal for the extraction of RNA. As is explained in the "Results" section, ICA is not a suitable extractant for DNA.

Methods of Estimation:

Estimation of Phosphorus

Total phosphorus was measured by a modification of the method of Griswold, Hummiller and McIntyre (1951), or by a modified Allen method (1940), using one-fifth quantities of the reagents.

1) Method of Griswold et al:

The sample (containing about 2 µg. P) was digested with a mixture of 4N ICA and 10N H_2SO_4 , cooled and diluted with water. The following reducing agent was then used:-

2.0 g. Na metabisulphite

4.0 g. $\text{Na}_2\text{S}_2\text{O}_3 \cdot 7\text{H}_2\text{O}$

and 1.0 g. 2-naphthol, 1-aminobenzene-4-sulphonic acid

dissolved in water and the volume made up to 1 litre.

To each tube was added 0.5 ml. of this solution and 0.5 ml. of ammonium molybdate solution (2.5% w/v), and the mixture was heated for 10 minutes in a boiling water-bath. Standard samples containing 2 μg (as Al_2O_3), and blanks containing the digestion acids only, were similarly digested (although digestion is not strictly necessary) and treated with reducing agent and molybdate. The tubes were cooled and their optical density was read in the 5100 spectrophotometer at 820 m μ against the reagent blank. If the colour was too strong, the solution could be diluted provided the concentration of acid was kept at 1N.

2) Method of Allen (modified):

Larger quantities of P could be estimated by a modification of Allen's method, using one-fifth of the quantities of reagents originally described. The samples, and standards containing 20 μg , were digested with 0.4 ml. 12N H_2SO_4 , cooled and treated with 0.4 ml. 1 acidol in 20. Na metabisulphite, followed by 0.2 ml. 5.5% ammonium molybdate. The mixture was diluted with 4 volumes of water and its optical density was read against a reagent blank at 633.6 m μ .

Estimation of ribonucleic acid

1) by the orcinol method:

The RNA content of the specimens was estimated by the following

modification of the orcinol method for ribose, based on that of Kerr and Seralidarian (1945), using a thirty minute heating period instead of their twenty minute period (Antoni, Ramaiah and Hutchison, in preparation).

The orcinol reagent was freshly prepared each day by dissolving 60 mg. crystalline orcinol per 10 ml. of 0.02% (w/v) solution of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (Analar) in conc. HCl (Analar). The standard used was a solution of D-ribose containing 10 μg per ml. Our calculations of RNA-P were based on the factor

$$10 \mu\text{g. ribose} \equiv 4.16 \mu\text{g. RNA-P}$$

This factor was obtained by the use of a standard preparation of yeast RNA, but the factor for rat liver RNA was later shown to differ by less than 5%. The colour given in the orcinol reaction by RNA depends chiefly on the purine-bound ribose, but about 12% is contributed by the pyrimidine-bound residues.

Method: To a 3 ml. sample containing 3-5 μg . RNA-P was added 3 ml. of orcinol reagent, and the solution, well mixed, was heated for thirty minutes in a boiling water-bath. The standard contained 1 ml. of the standard ribose solution + 2 ml. of water. After the colour had developed, the tubes were cooled and their optical density was read at 665 m μ against a reagent blank.

Note: Orcinol reacts with a variety of substances other than ribose and its compounds, and results obtained by this method may be too high. In general we found that it gave values a little lower than those obtained for the same specimens by a "total P" method, as would be expected, since

the latter method also measures "concomitant" P (Davidson and Smellie, 1952; Hutchison et al, 1956). The orcinol method is liable to interference by glycogen (as has been reported by Waterlow and Weiss, 1956), and it is known that a fraction of tissue glycogen is not removed by acid extraction (Stetten et al, 1958). Our animals, however, had usually been fasted for 18 hours before the experiment, so that the liver would be depleted of glycogen. Interference by sucrose has also been reported, e.g. by Slater (1958) and we have ourselves found that it causes black specks (presumably of carbon) and a brown interfering colour. The orcinol method is therefore not suitable for the estimation of RNA in cell fractions unless all traces of sucrose used in the fractionation procedure have been removed.

2) By the UV absorption method:

The RNA content of specimens containing sucrose may be estimated by the method of Fleck and Munro (1961), by measurement of their ultraviolet absorption at two wavelengths, but it should be remembered that this method is designed for an alkaline digest which has been incubated for a short time only. Its use for a specimen digested in alkali for 16 hours is unjustifiable, because the contribution to the absorption of the digested protein is then excessively large. If the UV absorption method is to be used, the digestion period must be shortened to 1 hour.

Estimation of deoxyribonucleic acid

The DNA content of whole alkaline digest, of the acid precipitable fraction of the alkaline digest, or of the LOA extract, was estimated by the method of Ceriotti (1955). In accordance with Ceriotti's modification

of his original method (personal communication), Analar chloroform was used without further purification. The standard used was departmental stock calf thymus DNA prepared by the method of Dounce, Simmons and Kay (1951). The standard solution was prepared by dissolving 20 mg. pure dry DNA in distilled water with a drop of alkali, and making up the volume to 50 ml. One ml. of this stock solution was diluted with 0.5N PCA, heated for 20 minutes at 70°, and made up to 50 ml. with 0.5N PCA. The standard DNA should therefore be comparable in reactivity to DNA which has undergone the Schneider extraction procedure. The Ceriotti procedure was also applied, however, to the acid-precipitated fraction of the Schmidt-Thamhauser alkaline digest obtained from liver and spleen. The results, calculated on the basis of the PCA-treated standard, were in good agreement with the P content. Moreover, our experiments on the extraction of DNA at 70° (described in the "Results" section) indicate that PCA causes significant degradation of deoxypentose only at concentrations greater than 2N. It was therefore concluded that the use of this standard in the estimation of the alkaline digest DNA was unlikely to introduce any serious error.

Estimation of nitrogen

All fractions were analysed for nitrogen by means of either a modified Nessler procedure or the micro-Kjeldahl method.

1) By the Nessler method:

The method was based on that of Paul (1958), which makes use of gum acacia to stabilise the Nessler reagent. The digestion of specimens was

carried out with 50% H_2SO_4 containing 1% selenium dioxide as a catalyst. A calibration curve was prepared by digesting different amounts of a standard ammonium sulphate solution with the acid mixture. The volumes of the standard solution were chosen to give a range of 2-30 $\mu g.$ N in 2 ml. of the final diluted digest, and a blank containing acid mixture only was included in the series. The modified Fessler's reagent (2 parts) and 2N NaOH (3 parts) were mixed immediately before use, and 5 ml. of the mixture were added to each 2 ml. sample and mixed well. After 10 minutes, the colour was read at 490 m μ against the reagent blank. The curve was found to be linear over the range studied, and for routine use a standard sample containing 20 $\mu g.$ N in 2 ml. of diluted digest was used. To economise on material, the method was frequently modified as follows; A sample expected to contain about 50 $\mu g.$ N was digested with 0.25 ml. (instead of 0.5 ml.) of 50% H_2SO_4 /1% SeO_2 mixture, cooled and the volume made up to 5 ml. (instead of 10 ml. as in the original method). A 2 ml. sample was then taken for estimation in the usual way. (Further saving could be achieved where necessary by taking only an expected 20 $\mu g.$ N and making the digest up to a final volume of 2 ml., provided the amount of acid was reduced proportionately.)

2) By the micro-Kjeldahl method:

Larger quantities of nitrogen could be estimated by a form of the micro-Kjeldahl procedure (Ma and Zuazaga, 1942). A pin-head of mercury was used as a catalyst in the digestion of the samples with concentrated nitrogen-free H_2SO_4 . The ammonia liberated by subsequent treatment with

10N NaOH was trapped in 2% boric acid, and titrated against 0.01N HCl. The titration was carried out with Ma and Zuazaga's indicator. A solution of ammonium sulphate, similarly treated, was used as a standard, and the titration values were corrected for a reagent blank.

In both methods of estimating N, it is important to avoid contamination of the solutions, especially the acid, by atmospheric ammonia. Such contamination was minimised by ensuring that no ammonia was stored, or had been used during the previous few days, in the room where the estimations were carried out.

Estimation of protein

1) By the Lowry procedure:

The modification of the biuret reaction proposed by Lowry et al (1951), in which colour development depends on the phenolic amino acids in protein as well as on the peptide bonds, was used as a rough guide to the protein content of samples. Since the colour developed in this reaction by a particular protein depends on its amino acid composition, the standard used in the preparation of a calibration curve should be a sample of the protein to be estimated. As no purified preparations of our liver proteins were available, our estimations were made on the basis of a calibration curve made by the use of bovine serum albumin. Thus the values obtained are suspect, but they may be used for comparisons of amounts provided such comparisons are made only within one type of protein, e.g. for comparisons of the specific activities at different times of similar preparations. Moreover, as will be seen later, the results of paper

chromatography indicate that the amino acid composition of the material for which this method was used is not grossly different from that of serum albumin. In a few cases, specimens were analysed by both the Lowry and the micro-Kjeldahl methods, and it was found that, for the protein left in the tissue residue after extraction by lipid solvents, the two methods gave very similar results (the micro-Kjeldahl value, corrected for nucleic acid N, was 4-5% higher than the Lowry value, if we assume a nitrogen content of 16% in the protein). The protein extracted by the lipid solvents gave a N figure by the Lowry reaction about 8% higher than that given by the micro-Kjeldahl method (corrected for phospholipid N).

A check carried out on the technique of the reaction showed that although, as Lowry et al point out, it is necessary to mix the solution very rapidly after the addition of the Polin-Ciocalteu reagent (this is most easily achieved by blowing the reagent in from a graduated pipette), the time allowed to elapse between the addition of the alkaline copper reagent and the addition of the Polin-Ciocalteu reagent does not seem to be very critical. Lowry et al mention 10 minutes, but 5 minutes seem to be sufficient and 15 minutes is not too long. The time allowed between the addition of the Polin-Ciocalteu reagent and the measurement of the optical density also appears not to be critical, as the reading was the same 25, 30 and 45 minutes after addition of the reagent.

In this method of estimation, the calibration curve used was not strictly linear, although it was approximately so up to an O.D. of 0.35. It was therefore necessary to read off the amount of protein in each sample

from the graph, instead of calculating it on the basis of a standard tube included in each series.

Although the Lowry reaction is carried out in aqueous solution, it was found to be easily applicable to the organic solvent extracts. The extracts were diluted with a large excess of water (generally 100-fold), so that the sample taken for estimation contained very little organic solvent. Any faint cloudiness which appeared on dilution with water disappeared when the alkaline copper reagent was added.

2) By the Biuret method:

The modification used was that proposed by Gornall, Bardawill and David (1949) for the measurement of plasma proteins. As in the case of the Lowry form of this reaction, it was necessary to use a calibration curve based on an arbitrarily chosen standard protein. In this case, two proteins, egg albumin and casein, were used. Various volumes of a 0.5% (w/v) solution of each protein (in approximately 0.05N NaOH) were taken, over the range 1-10 mg. protein, and estimated by the biuret reaction. When the O.D. at 540 mμ was plotted against the protein content, the two proteins gave almost identical, linear curves, which were then used to provide a very rough idea of the protein content of the lipid solvent extract of liver. Samples of lipid extract containing an expected 5 mg. protein were evaporated almost, but not quite, to dryness and made up to 2 ml. with distilled water. When the biuret reagent was added, the lipid extract material redissolved, and after 30 minutes the colour was read at 540 mμ.

Amino-N estimation by the ninhydrin method:

The method used was that of Yemm and Cocking (1955). For purposes of rough comparison, calibration curves were constructed for egg albumin and for casein as follows. Portions of 0.1 ml. of the 0.5% solutions in NaOH already used for the biuret estimation were hydrolysed for 18 hours at 115°C with 0.3 ml. 8N HCl in a sealed tube. After removal of excess HCl in a vacuum desiccator over $P_2O_5/NaOH$, the volume was made up to 10 ml. with water. Curves were constructed over the range 0.5-25µg. protein, using different volumes of the diluted hydrolysates, and the O.D. at 750 mµ was plotted against the N content of samples. (The N content of the standard proteins was known). It was found that the two proteins gave almost identical curves.

The lipid solvent extracts were prepared for estimation as follows. Portions of extract containing about 150 µg. N were dried in air at room temperature, and hydrolysed with 1 ml. 6N HCl at 115°C for 16 hours. The hydrolysates were dried, the HCl removed in a vacuum desiccator over $NaOH/P_2O_5$, and the residue taken up in 0.1 ml. of water. 25 µl of this solution was diluted to 50 ml. and 1 ml. and 2 ml. portions were estimated by the ninhydrin method.

Extraction of lipid extract with water (Folch procedure):

The lipid solvent extract of TCA-precipitated liver was treated with water by the method described by Folch, Lees and Sloane-Stanley (1957) for the fractionation of chloroform/methanol tissue extracts. The procedure adopted for chloroform/methanol extracts was as follows: a 4 ml. portion

of lipid extract (generally derived from the microsomal pellet from 200 mg. liver) was thoroughly mixed with 0.8 ml. of distilled water at room temperature and spun for a few minutes to give good separation of the phases. A whitish mat separated at the interface, and could be lifted out on a glass rod after removal of the lower (organic) phase by means of a Pasteur pipette. In the case of polar solvent extracts, e.g. acetone or ethanol, it was necessary to evaporate the 4 ml. sample of extract to small bulk, under reduced pressure, and make the volume up again to 4 ml. with chloroform/methanol (2:1), before adding the water. Folch et al recommend the addition of salts (e.g. CaCl_2) to the washing water to prevent the loss of lipid material into the aqueous phase. We did not add salts, since our interest lay not in the lipids but in the protein material which Folch et al were attempting to remove from the lipid extract, and this, we found, separated under our conditions at the interface. Folch et al did not obtain any fluff by this procedure, but their extract may differ from ours, since they homogenised the tissue directly in chloroform/methanol. It is interesting that in earlier experiments (1951), Folch and Lees found that a similar chloroform/methanol extract did give interfacial fluff when washed with a large volume of water. This "fluff" they found to be a mixture of proteolipids, a type of lipoprotein insoluble in water.

Paper chromatography:

Paper chromatography of the hydrolysed lipid extract was carried out on the same hydrolysate as was used for the quantitative ninhydrin reaction. From what remained of the 100 μl . of hydrolysate, portions of 30 μl .

(containing about 40 μ g. N) were applied to 57 x 46 cm. sheets of Whatman No. 1 chromatography paper and run for 18 hours, by descending chromatography, in the organic layer of a mixture of n-Butanol/acetic acid/water (4:1:5). After thorough drying in warm air, the paper was run in the second dimension, by the ascending technique, in a phenol/water/ammonia solvent, for 18 hours. The paper was again dried in warm air, dipped in 0.5% ninhydrin in acetone, and heated at 90°C for 5-10 minutes until the colour developed. The paper was sprayed with zinc sulphate solution to make the spots permanent.

Equivalent amounts of the unhydrolysed material were similarly treated, and a hydrolysate of egg albumin was also run as a reference chromatogram to help in the identification of amino acids.

Column chromatography:

A small column (8 cm. x 1.1 cm²) of DEAE cellulose was prepared according to the method of Sober et al. (1956), and washed with 0.005 M Na₂HPO₄. Portions of 2 ml. of lipid extract, containing about 10 mg. of protein, were applied to the top of the column and washed through with about 30 ml. of 0.005 M Na₂HPO₄ (pH 7.6). A brownish band was seen to move about 5 mm. down the column, and thereafter to remain almost stationary. Attempts were made to elute the proteins or peptides of the lipid extract with phosphate buffers of decreasing pH and increasing ionic strength (from pH 7.6 and molarity 0.005 to pH 4.1 and molarity 0.02), and finally with buffers of high ionic strength containing sodium chloride also. Alkaline buffers were not used. The fractions emerging from the column were examined for

protein by measurement of their UV absorption spectrum and by the Lowry reaction.

Paper electrophoresis:

Paper electrophoresis of the lipid extract material was carried out on Whatman No. 1 paper cut in strips, usually about 40-50 cm. long and from 4 to 9 cm. wide. The method used was that described by Poulik and Smithies (1958). Portions of lipid solvent extract containing about 700-800 µg. protein were applied along a line about 6 cm. from the cathode end of the paper, a margin of 5 mm. being left at each side. The strip was then run for 17 hours in a current of 3 volts per cm. in the following buffer (of pH 8.6):-

Sodium acetate 0.048 M	}
Sodium barbital 0.048 M	
Hydrochloric acid 0.0073 M	

The paper was allowed to equilibrate for 30 minutes before the current was switched on, and wicks of 3 MM. paper were used to dip into the electrode vessels. After electrophoresis, the strip was dried at room temperature and cut lengthwise into either two or three strips. The individual strips were then examined for protein and lipid material, and where appropriate for radioactivity, as follows:

1) Detection of protein:

The strip was heated at 120° for 30 minutes in order to coagulate the proteins. It was then stained with bromophenol blue according to the method described in the Spingo Analytrol manual (procedure B for serum proteins).

2) Detection of lipid:

It was found that Sudan IV, the first stain for lipid tried, was too insensitive, and the stain eventually used was Sudan Black, according to a modification of the method of Dangerfield and Smith (1955). The paper was stained for 30 minutes in a saturated solution of Sudan Black in 55% ethanol, which had been matured for a few days and then filtered. The paper was quickly rinsed in 50% ethanol and thoroughly washed in 40% ethanol until the background was reasonably clear (though not colourless).

3) Detection of radioactivity:

In experiments in which the proteins had been labelled with radioactive leucine, the strips were examined for radioactivity by means of the Actigraph (Nuclear Chicago) automatic scanner, set at its maximum sensitivity, the counts being integrated over a period of 4 seconds, since the activity of most of the strip was found to be very low.

Injection of radioisotopes in vivo:

Carrier-free ^{14}C -(DL)-leucine, obtained in the solid form from the Radiochemical Centre, Amersham, was dissolved in distilled water and diluted to give a solution containing 50 μC per ml. This solution was stored at -5° until the day of the experiment. It was then diluted with sodium chloride solution to give a 0.9% (physiologically normal) saline solution containing 25 μC per ml. Half a ml. of this solution was injected via the tail vein into each rat under ether anaesthesia. Injection was made easier by heating the tail in a beaker of hot water for a few moments beforehand. The injections were performed by Dr. A. Fleck and Dr. P. Goswami.

In experiments designed to follow the incorporation of the labelled amino acid within a short time of injection, it was found possible to keep the rat alive for up to 15 or 20 minutes under light anaesthesia. In this way, single lobes of the liver could be removed at different time intervals after the injection.

Counting of radioactive samples:

On one occasion counting was carried out in an end-window counter on a sample of the acid-insoluble, lipid-free residue from the tissue, as a dry powder at infinite thickness, but in general counting was carried out by means of a gas-flow counter (efficiency about 24%). Samples were plated on single thicknesses of lens tissue on aluminium planchettes (5.15 cm.²), and dried under an infra-red lamp. Counting was carried out for 500 or 1000 counts, and the background count (usually 23 counts/minute) was subtracted. The counts were not high enough to require correction for "dead time".

Tests were carried out on different amounts of labelled protein plated on lens tissue as described, and counted (a) by means of an end-window counter and (b) in the gas-flow counter. By both methods, it was found that the "specific activity curve" was linear up to 10 or 15 mg. protein (applied in a volume of 0.10 or 0.15 ml.). This implies that self-absorption is constant by this technique and does not increase with increasing amounts of material, as in the case of protein powder.

Chemical treatment of the microsomal fraction:

Various reagents were used to bring about differential breakdown of the microsomes. Pyrophosphate was employed to disintegrate the ribosomal granules and leave intact the membranous component, whereas deoxycholate (DOC) was used to remove the membrane. Other reagents used in attempts to disintegrate the microsomes were lysollecithin, and a bacterial toxin (prepared from Staph. aureus).

(a) Pyrophosphate treatment:

"Heavy" and "Light" microsomal fractions were prepared according to the scheme shown in Fig. 5, and the "heavy" microsomes were resuspended in sucrose solution and again spun down at 12,000 g for 60 minutes. All pellets were then homogenised in 10 ml. of the sodium pyrophosphate (PP) reagent described by Sachs (1957, 1958), i.e. a 0.11% solution of $\text{Na}_2\text{P}_2\text{O}_7$ in 0.25 M sucrose, adjusted to pH 7.4 by the addition of HCl. Sucrose solution was added to fill the tube, so that the final concentration of pyrophosphate was about 0.10%. All operations were carried out at 0°C. The suspensions were centrifuged for 45 minutes at 105,000g, either immediately or after a period of incubation at 0°C.

The resulting "P_i-soluble" (ribosomal) and "P_i-insoluble" (membranous) fractions were then precipitated with TCA and extracted as usual with lipid solvents. Phospholipid analysis was not possible in the case of the "P_i-soluble" fractions, since the pyrophosphate interfered with the estimation of phosphorus.

(b) Deoxycholate treatment:

The method used was based on that described by Littlefield, Keller, Gross and Zamecnik (1955), and was applied to a microsomal pellet prepared by their cell fractionation procedure. The liver was homogenised in 3 volumes of ice-cold 0.25 M sucrose in a Potter homogeniser. After removal of whole cells, nuclei and mitochondria by centrifuging for 10 minutes at 18,000 g, the supernatant was spun for 45 minutes at 105,000 g. The pellet was drained, and the inside of the tube carefully blotted dry. Each tube usually contained the microsomes from about 2.5 g. of whole liver. It should be noted that this method of preparation gives a fraction which contains "light" as well as "heavy" microsomes, although it may not be completely identical with the combined "heavy microsome" and "light microsome" fractions obtained by the scheme shown in Fig. 5.

A cold 5% solution of sodium deoxycholate (DOC) in 0.2 M glycylglycine buffer (adjusted to pH 8.0 by addition of NaOH) was prepared freshly before use. Each pellet was carefully ground up with 0.8 ml. of this solution, by means of a thick round-ended glass rod or a Potter homogeniser pestle, until no gross particles remained. The tube was kept chilled throughout, and was then allowed to stand for 5 minutes in ice. The tube was then filled with ice-cold water, and spun at 105,000 g for 2 hours. The supernatant (the DOC-soluble fraction) was decanted off, the last 0.5 ml. being left with the DOC-insoluble pellet. The fractions were then precipitated and washed with TCA, and where appropriate extracted with lipid solvents, in the usual way.

(c) Lysolecithin treatment:

The lysolecithin was supplied by Dr. C. Long, who prepared it from ovoidlecithin by the action of cottonmouth moccasin venom according to the method of Long and Penny (1957). The ovoidlecithin itself was prepared from hens' eggs by the procedure of Rhodes and Lea (1957). The lysolecithin was dissolved in chloroform/methanol (96:4 v/v) at a concentration of approximately 40 mg. per ml., and was stored at -5°C . It became insoluble during storage at low temperature, but redissolved at room temperature.

An experiment performed to discover a suitable quantity of lysolecithin for use with microsomal pellets prepared from 1 g. of liver indicated that 10 mg. per pellet had a greater effect than 5 mg. and was not much less effective than 20 mg. We therefore used 10 mg. in most of our experiments until shortage of lysolecithin caused us to reduce the quantity to 5 mg.

The following procedure was adopted for mixing the lysolecithin with the microsomal pellet. A volume of lysolecithin solution (equivalent to 5 or 10 mg.) was pipetted into a clean test-tube, and the small volume of solvent evaporated almost, but not quite, to dryness. Five ml. of 0.2 M glycyl-glycine buffer (pH 8.0) were added, and the tube was well shaken. The microsomal pellet which was to be treated was suspended in 3 ml. of 0.25 M sucrose at 0° , and the 5 ml. of lysolecithin in glycyl-glycine buffer was added. The lysolecithin test-tube was rinsed out with a further 3 ml. of 0.25 M sucrose solution, which was added to the pellet suspension. The tubes were filled up with 0.25 M sucrose solution and kept at 0°C . for 48 hours. They were then spun at 105,000 g for 1 hour. The pellets were

precipitated with TCA and extracted as usual with lipid solvents. The same procedure was used for both "heavy" and "light" microsomes.

(d) Haemolysin:

This toxin was prepared from Staphylococcus aureus by Dr. I. R. W. Lominski, who uses it to lyse red cells. A specimen of the toxin which had undergone autoclaving for 30 minutes, and which was no longer capable of attacking red cell membranes, was used as a control. In the first experiment, the microsomal pellet was prepared in sucrose as usual, and incubation with toxin, carried out in sucrose solution, appeared to have no effect on the pellet. It was then found that the haemolytic action of the α -toxin was inhibited by sucrose, and it seemed possible that sucrose was also inhibiting any action it might have on the microsomes. In subsequent experiments, therefore, the pellet, after preparation in sucrose, was washed with Krebs-Ringer bicarbonate solution, and the incubation with the toxin was also carried out in the bicarbonate buffer solution. Each pellet was suspended in 2 ml. of buffer, and treated with 1 ml. of active or heated toxin at 37°C for 1 hour. "Blank" tubes were incubated without microsomes, so that the protein or other content of the toxin preparation could be corrected for. After incubation, the tubes were chilled, filled up with sucrose solution and centrifuged for 1 hour at 18,000 g at 0°C, to spin down the undissolved microsomes. As usual, the pellets were analysed by precipitation with TCA and extraction with lipid solvents.

Electron microscopy:

Small pieces of liver, about 0.5 mm. thick, or portions of microsomal

or ribosomal pellets prepared in 0.24 M sucrose as described above, were removed by means of a razor blade or scalpel and dropped into a 2% solution buffered of osmic acid (of pH 7.2). After fixation for 2 or 3 hours the specimens were thoroughly washed, dehydrated in alcohol and embedded in methacrylate. Sections were examined at a magnification of 2000-3000, usually in a Phillips electron microscope. Better resolution could be obtained by staining with uranyl acetate and using a Siemens electron microscope.

RESULTS

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The objects of the investigation have already been outlined in the introduction. The first was to determine the quantity of organic nitrogen, other than phospholipid nitrogen, which is removed from whole liver by the different solvents used to extract fats and phospholipids. Since this amount was found to be quite large, the chemical nature of the material was explored, and, by means of ultracentrifugal and chemical techniques, its distribution within the cell was studied. The ability of the fraction to incorporate radioactive amino acids was compared with that of other protein fractions of the cell, and investigations were carried out on the effect of variations in diet on this metabolic activity, as well as on the actual amount of material.

SECTION I

Quantitative estimation of "lipid N" in Liver

In many procedures for the analysis of tissues it is common practice to extract the lipids of the preparation with organic solvents. For this purpose a variety of solvents has been used, often at room temperature and sometimes at higher temperatures. Fig. 6 gives the outline of a scheme which is frequently used. Such extraction methods are employed in the Schneider and Schmidt-Thannhauser procedures, which have been adapted to measure the protein content of tissues as well as their nucleic acids.

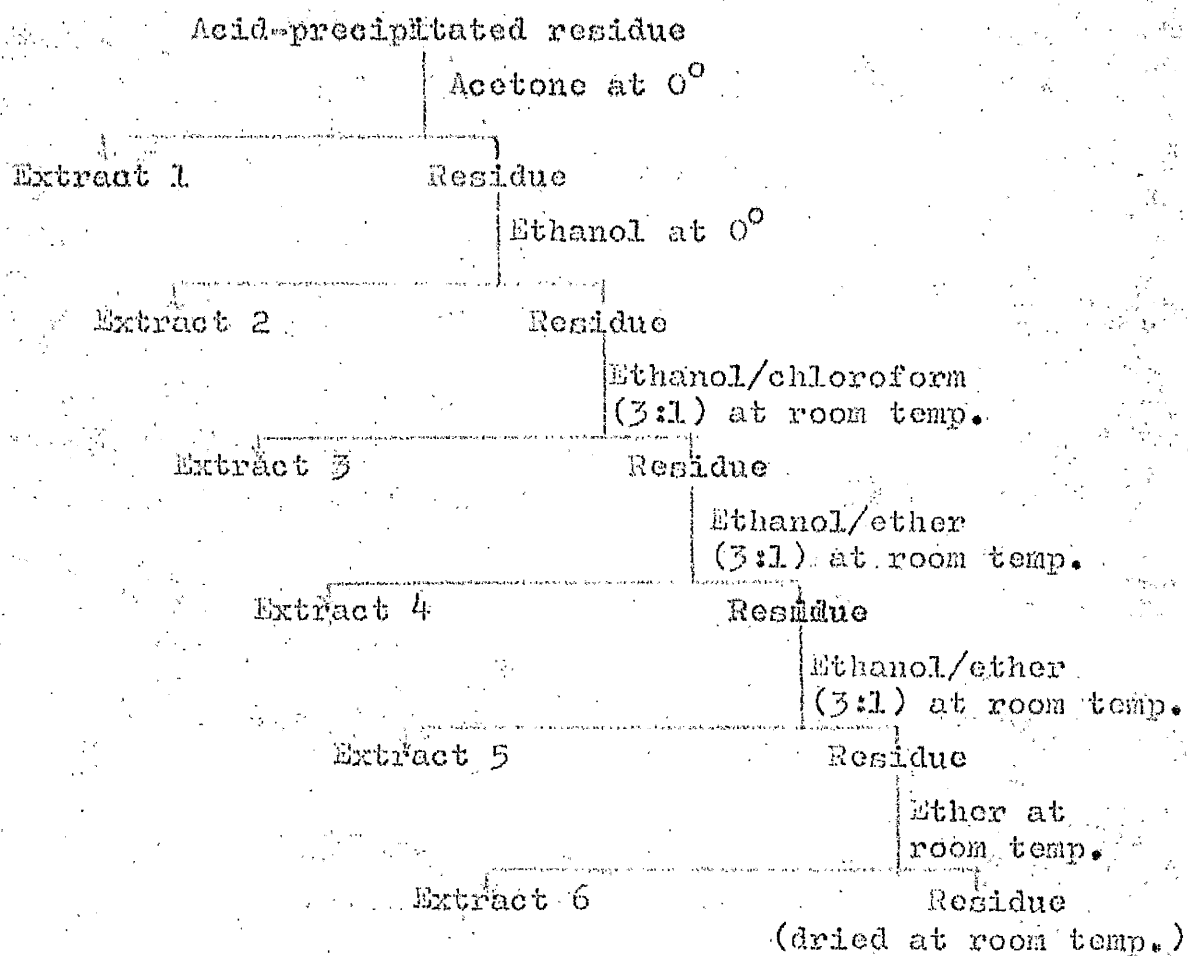
The assumption underlying these methods is that the lipid solvent treatment will remove only fats and phospholipids. If this is true, then the nitrogen appearing in the lipid solvent extract will be contributed by the nitrogenous bases of the phospholipids and will include no protein N or nucleic acid N.

One method, therefore, of tackling the problem is to estimate the N and P of the lipid solvent extract, and, assuming that this P is entirely phospholipid P, to calculate the excess N present. If this excess is appreciable, then the possibility of loss of protein and/or nucleic acids must be considered.

An alternative approach is to examine the residue left after lipid solvent treatment, and determine whether it is depleted of protein and nucleic acids compared with specimens of the tissue not subjected to lipid solvent treatment. In order to obtain a measure of the protein N content of the tissue residue, several estimations have to be made. The nitrogen

FIGURE 6

Extraction with lipid solvents



comes from (a) protein, (b) the purine and pyrimidine rings of the RNA and DNA of the tissue residue, and (c) phospholipid, where this has not been extracted by lipid solvents. It is therefore necessary to estimate the total N, total P and, by appropriate specific colour reactions, the nucleic acid content of the residue.

The estimates of "lipid N" obtained by these different approaches can then be computed as follows:

A. From the analysis of the lipid solvent extract:

$$\text{"Lipid N"} = \text{total N of the extract} - \text{phospholipid N} \dots (1)$$

$$\text{where phospholipid N} = \text{total P of the extract} \times 0.45$$

B. From analysis of the tissue residue:

$$\begin{aligned} \text{"Lipid N"} = & \text{protein N of the unextracted residue} - \\ & \text{protein N of the extracted residue} \dots (2) \end{aligned}$$

The estimates of "protein N" are obtained as follows:

$$\text{protein N} = \text{total N} - (\text{nucleic acid N} + \text{phospholipid N})$$

By applying this equation to the extracted and unextracted residues we obtain the following relations:

$$\begin{aligned} 1) \text{ Protein N of the extracted residue} = & \text{total N of extracted residue} - \\ & \text{nucleic acid N of extracted residue} \dots (3) \end{aligned}$$

$$\text{where nucleic acid N} = \text{nucleic acid P} \times 1.69$$

$$\begin{aligned} 2) \text{ Protein N of the unextracted residue} = & \text{total N of unextracted residue} - \\ & (\text{nucleic acid N of unextracted residue} + \text{phospholipid N} \\ & \text{of unextracted residue}) \dots (4) \end{aligned}$$

$$\text{where nucleic acid N} = \text{nucleic acid P} \times 1.69$$

$$\text{phospholipid N} = \text{phospholipid P} \times 0.45$$

$$\begin{aligned} \text{and phospholipid P} = & \text{total P of unextracted residue} - \\ & \text{total P of extracted residue} \dots (5) \end{aligned}$$

Notes:

(1) The following factors are used:

$$\text{RNA-P} = \text{ribose (by orcinol)} \times 0.416$$

$$\text{DNA-P} = \text{DNA} = 0.096$$

$$\text{nucleic acid N} = \text{nucleic acid P} \times 1.69$$

$$\text{(i.e. ribose} \times 0.70 \text{ and DNA} \times 0.162)$$

$$\text{phospholipid N} = \text{phospholipid P} \times 0.45$$

$$\text{phospholipid} = \text{phospholipid P} \times 22.7 \text{ (Artom and Fishmann, 1943)}$$

(2) Method A assumes that all the lipid extract is phospholipid P. This assumption may be unjustified if the solvents also extract nucleic acids. Hence the effect of lipid solvents on nucleic acids must be tested, and the results of such tests will be given in Section IIA.

(3) Method B assumes that all the phospholipid is extracted by the lipid solvent (equation 5). The conclusions reached by Hutchison and Munro (1961) indicate that this assumption is probably justified.

(4) In estimates of "lipid N" obtained by method B (analysis of the residue), it is possible to calculate the N of the residue as "protein N", i.e. corrected for nucleic acid N (equations 3 and 4). Thus the estimate of "lipid N" based on this method of calculation will not include any nucleic acid which may be extracted by the lipid solvents (equation 2).

Correction for nucleic acid is not possible in method A (analysis of the solvent extract), and the estimate obtained by that method may include nucleic acid N (equation 1). If the solvent extract does contain nucleic acid, then as explained in Note 2, the figure for phospholipid N in the solvent

extract will also be too high. This error will help to reduce any spuriously high figures for "lipid N" caused by extraction of nucleic acids.

On occasion, both methods were used in the same experiment, and it was then possible to compare the results obtained directly, by analysis of the lipid solvent extract, with those obtained indirectly by analysis of the extracted and unextracted residues. The agreement was generally good.

Table 3 shows the results of a series of experiments in which method B was used. Portions of whole homogenate of rat liver were precipitated and washed with TCA, and then treated with either (a) the full series of lipid solvents, (b) the full series except for acetone (the first solvent) or (c) with sodium hydroxide only (sufficient to neutralise the residual TCA). The residue was then examined for total N, nucleic acid and total P.

There is a loss of about 22% of the total N in the lipid-extracted specimens, and of 50% of the total P. Even if it is assumed that all of the lipid P is in the form of phospholipid, the nitrogen equivalent of the lost P is only about 50 µg. N per 100 mg. liver, a figure which accounts for a mere 10% of the N lost.

The results also indicate that the omission of acetone from the series of solvents has little effect on the amount of N or P removed. This suggests either that acetone is just as efficient a solvent as ethanol, or that the removal by acetone of most of the residual TCA from the acid-precipitated material does not prevent the loss of "lipid N" in ethanol and subsequent solvents.

TABLE 3

The effect of extraction by lipid solvents on the P and N content
of the acid-insoluble residue of rat liver

Treatment	Total P (μ g. per 100 mg. liver)	Total N (corrected for nucleic acid N) (mg. per 100 mg. liver)
Unextracted	230	2.47
Extracted without use of acetone	115	1.90
Extracted with all solvents	112	1.95

(mean of 4 experiments)

The data thus indicate a loss of acid-insoluble N other than phospholipid N into the lipid solvents. This could represent either nucleic acid N or protein N.

Having established that nitrogen other than phospholipid N is removed by the solvents used for lipid extraction, we next tried to determine the extent of this loss of N under different conditions. The first factor investigated was the time of exposure to the solvents. Table 4 and Fig. 7 display the results obtained in an experiment in which the time of contact with ethanol at 0°C was varied from 7 minutes (the minimum time for manipulation) up to 24 hours. The results demonstrate that the loss of N increases a little with the time of exposure up to 3 hours, and that the inclusion of acetone in the series may have a slight effect in accelerating this N loss.

The next question investigated was whether this extraction of non-phospholipid N was due to any single solvent in the series used for the extraction of lipids. Acetone, ethanol and ethanol/chloroform (3:1) were used separately to extract a preparation of rat liver microsomes which had been precipitated with TCA. On this occasion analysis was carried out on the lipid solvent extract rather than on the residue (Method A above). The nitrogen and phosphorus in the lipid extract were measured for each solvent. Table 5 demonstrates that nitrogen over and above the amount due to phospholipid was removed by each solvent. The table shows that on average acetone removed less "lipid N" than the other solvents tested, but the efficiency of acetone was found to be very variable. In some

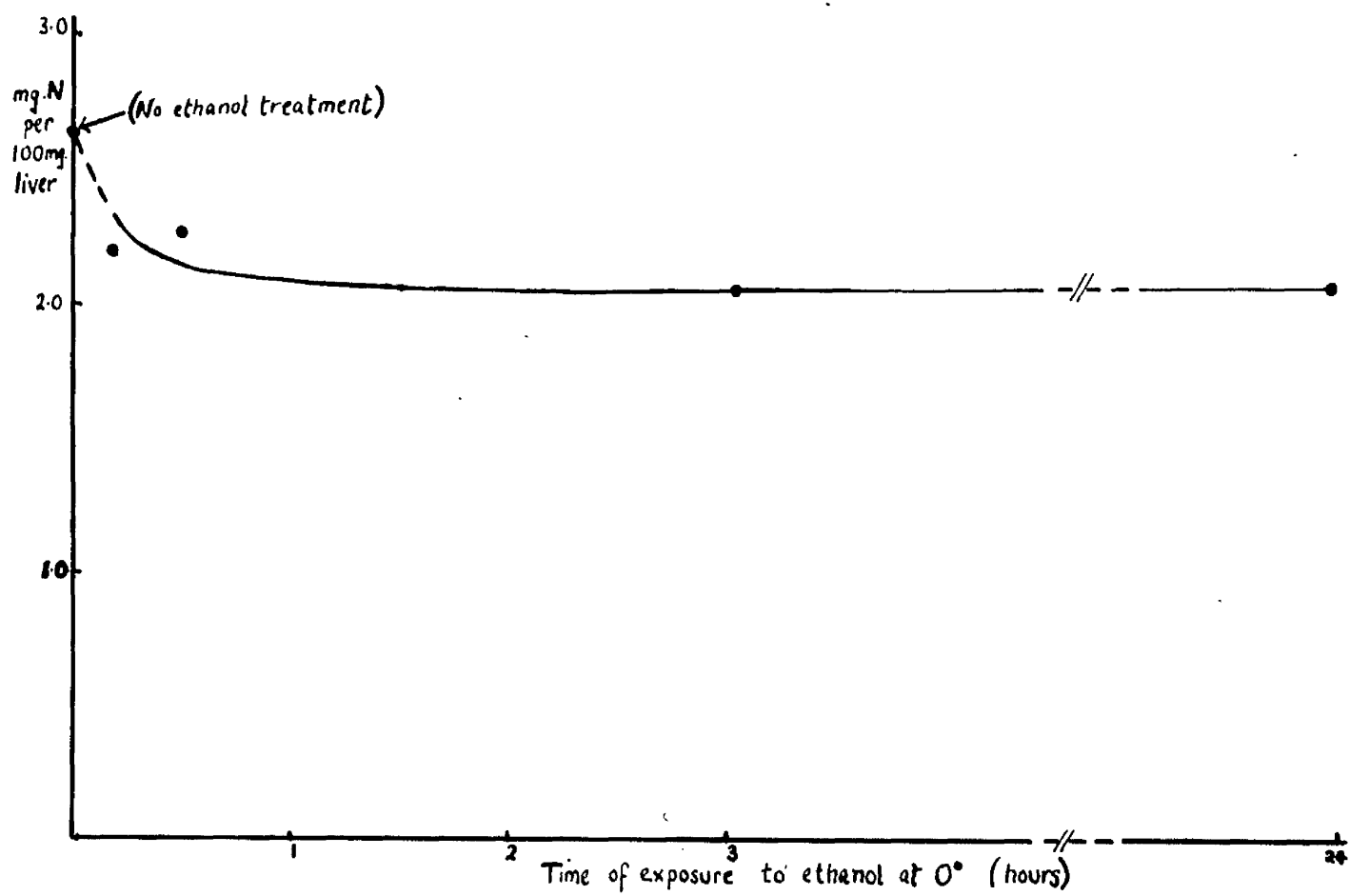
TABLE 4

Effect of exposure to ethanol for various times on the N content of
the acid-insoluble residue from rat liver

After precipitation by TCA, the test specimens were treated with ethanol at 0°C for the times stated. Acetone was not used except in the case of the last specimen. The results are expressed as mg. N in the residue per 100 mg. liver, and have been corrected for nucleic acid nitrogen.

Treatment	N of Residue	Difference from Control
Control (no solvents)	2.66	-
Cold ethanol for 7 minutes	2.20	0.46
" " " 1 hour	2.27	0.39
" " " 5 hours	2.06	0.60
" " " 24 hours	2.09	0.59
Cold acetone, then cold ethanol (each for 7 minutes)	2.11	0.55

FIGURE 7



Effect on "lipid N" of prolonged exposure to ethanol at 0°

TABLE 5

Efficiency of different solvents in removing "lipid N" from
rat liver heavy microsomes

Each microsomal pellet was precipitated and washed with 10% TCA, and then extracted with a 5 ml. portion of acetone, ethanol or ethanol/chloroform (3:1), i.e. each solvent was the first applied after TCA treatment. In this experiment the N figures are calculated from the results of a Lowry protein estimation, on the assumption that the protein contains 16% N. (This method of estimation will be justified in Section IIB). The results are the mean of those obtained for 4 homogenates.

	ug. "lipid N" of microsomes from 100 mg. liver
acetone	63
Ethanol	104
Ethanol/chloroform	102

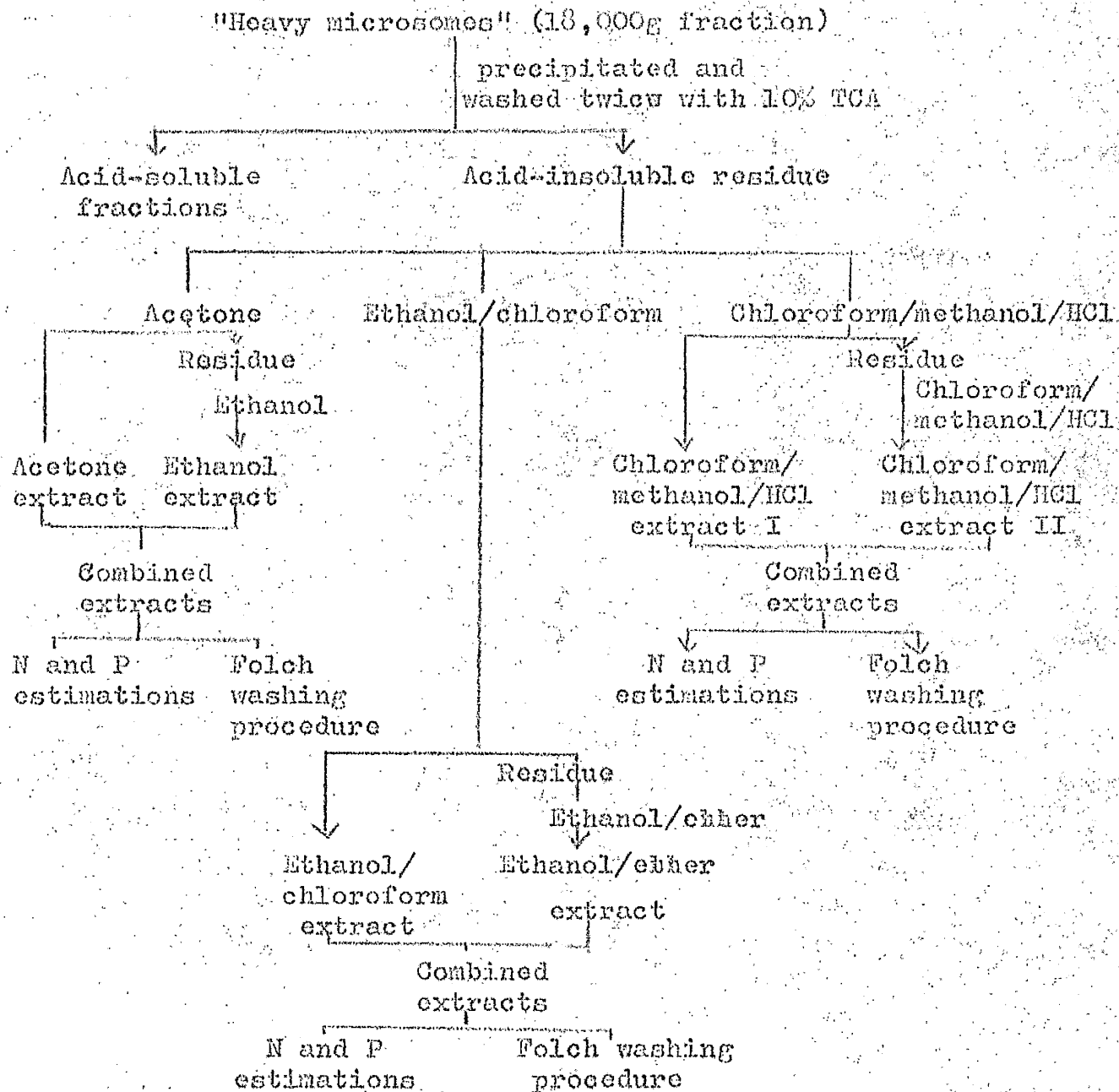
experiments it removed much the same amount of "lipid N" as the other solvents, and in others only about half the quantity. This difference may reflect differences in the state of aggregation of the acid-insoluble residue. Where acetone removed only a part of the "lipid N", subsequent extraction with ethanol usually removed a further quantity. In general, however, after one solvent had been used, later treatment with other solvents removed very little additional "lipid N", a finding which suggests that each solvent removes the same material.

Another solvent which is sometimes used for the extraction of lipid material from tissue preparations, but which does not occur in our usual scheme (Figure 6), is a chloroform/methanol mixture, sometimes containing HCl (1 part in 300). Folch, Lees and Sloane-Stanley (1957) have shown that the phospholipid contained in such an extract may be separated from any protein-lipid complexes by washing with water. The first column of Table 6 shows the relative efficiencies as extractants of chloroform/methanol and some other solvents. The extracts were prepared as shown in the flowchart, Figure 8, from rat liver heavy microsomes precipitated with TCA. Only two solvents were used in each case - acetone and ethanol, ethanol/chloroform and ethanol/ether, or chloroform/methanol/HCl used twice - and the two extracts were combined for analysis and fractionation.

From analysis of these whole (unfractionated) extracts, it appears that acetone and ethanol are less efficient in extracting "lipid N" than the other solvents. It is not possible to say whether this finding is reproducible, since only one experiment of this type was performed.

FIGURE 8

Preparation of extracts referred to in Table 6



The extracts were then washed with water, by the procedure of Folch et al (1957). The chloroform/methanol/HCl extract was mixed with 0.2 vols. of water, and a white fluff separated at the interface. In order to carry out the water-washing procedure on the polar solvent extracts, it was necessary first to evaporate the extract to small bulk under reduced pressure, and to take it up in chloroform/methanol. When this solution was mixed with 0.2 vols. of water, it also developed a white fluff at the interface.

This material, which could be packed into a mat by centrifugation, was found (Table 6) to contain nearly all the non-phospholipid N of the lipid extract, while the P remained in the organic layer. The aqueous phase contained little N or P. The general pattern of the distribution of the phospholipid and the "lipid N" on washing with water is the same for the three pairs of solvents tested, and the small variations may be due to the technical difficulty of obtaining a clean separation of the liquid layers and the interfacial mat. At any rate, it is clear that, whichever solvent was originally used, washing with water removes virtually all the "lipid N" from the organic layer, without greatly affecting the phospholipid.

So far we have considered only extracts prepared from material already treated with TCA. The next step was to study the effect of lipid solvents on tissue not previously exposed to acid. Table 7 shows the result of extracting rat liver by homogenising it directly in chilled acetone in a Waring blender. Since the tissue tended to clump, the acetone extraction was not very efficient, and the second extraction, with ethanol, removed a considerable further amount of phospholipid. The figures in this table

TABLE 6

The extraction of "lipid N" and phospholipid from TCA-treated rat liver heavy microsomes by various solvents, and the effect of washing with 0.2 vols. water (Polch procedure)

The results in the first columns are expressed as $\mu\text{g. N}$ (corrected for phospholipid N) or $\mu\text{g. P}$ in the solvent extract of the microsomes derived from 100 mg. liver. The other results are expressed as a percentage of the N or P in the whole, unfractionated extract.

Solvents	Whole extract		Aqueous phase		Interphase		Organic phase	
	N	P	N	P	N	P	N	P
Acetone + ethanol	84.4	31.3	16%	5%	84%	5%	0%	90%
Ethanol/chloroform + ethanol/ether	173	40.9	10%	6%	88%	23%	2%	74%
Chloroform/ methanol/HCl (twice)	180	40.1	5%	14%	95%	10%	0%	77%

TABLE 7

Effect of lipid solvents on rat livernot previously treated with TCA

The liver was homogenised in chilled acetone (5 vols.) and the acetone extract was separated by centrifugation. The residue was then extracted with ethanol at 0°, and both extracts were analysed for total N and P. The non-phospholipid N content of the extract was calculated from these results. All the figures are expressed as µg. N or P per 100 mg. liver.

Extract	Total N	Total P	Non-phospholipid N
Acetone extract	43	43	24
Ethanol extract of residue	35	61	8
Total extracted	78	104	32

may be compared with those in Table 3, where the total P extracted by the series of lipid solvents after TCA treatment was 118 μ g. per 100 mg. liver and the total N 520 μ g. per 100 mg. liver. The corresponding figures when TCA treatment is omitted are seen to be 104 and 78 μ g. respectively. The total P extracted is thus of the same order as that removed by lipid extraction after TCA precipitation, but the non-phospholipid N is almost completely undissolved. The general pattern of the figures in the last column was confirmed by the Lowry reaction.

It follows from these data that the conditions required for the extraction of "lipid N" from whole rat liver homogenate differ from those which bring about the extraction of phospholipid. One possible reason for this difference is that in TCA-precipitated specimens the acid-soluble residue retains a small quantity of TCA, and the presence of acid in the lipid solvent may be necessary for the extraction of "lipid N", although not of phospholipid. If lack of acid is the reason for the failure of acetone and ethanol to extract "lipid N" from specimens not previously treated with TCA, then one would expect that an acid-containing solvent, such as the chloroform/methanol/HCl mixture used in the experiments described in Table 6, would be effective even without prior TCA treatment. (The acid content of the drained TCA-wet precipitate is probably similar to that of 5 ml. of chloroform/methanol/HCl). In Table 6, however, chloroform/methanol/HCl was shown to remove considerably more "lipid N" than acetone, and it is possible that the substances removed by the different solvents are not the same. We therefore carried out two experiments in which samples were

treated first with the usual series of solvents, and then with chloroform/methanol/HCl, to see whether the chloroform/methanol/HCl removed any further "lipid N" material. Similarly, samples extracted first with chloroform/methanol/HCl were then treated with the usual series of solvents. These procedures were carried out on both untreated and TCA-treated microsomal pellets, and the "lipid N" and phospholipid content of the various extracts were estimated. The results are given in Table 8.

Let us consider first the case where the samples were treated with TCA before extraction with lipid solvents. After extraction with the usual series of lipid solvents, extraction with chloroform/methanol/HCl removes very little further phospholipid (0.07 mg.). On the other hand, two extractions with chloroform/methanol/HCl, used as the initial solvent, remove the same quantity of phospholipid as in the first case, and subsequent treatment with the usual solvents removes very little more (0.04 mg.). Thus both treatments seem to be fully efficient in the extraction of phospholipid.

As was found earlier (Table 6), chloroform/methanol/HCl removes more "lipid N" (1.55 mg.) than the usual series of solvents (0.97 mg.), but only if it is used before the other solvents. Treatment with chloroform/methanol/HCl after extraction with the usual series removes very little further "lipid N" (0.03 mg.). Conversely, after initial chloroform/methanol/HCl treatment, the usual solvents remove very little "lipid N" (0.08 mg.). It thus appears that the usual solvents are less efficient than chloroform/methanol/HCl in removing "lipid N", but that after their use the "excess" "lipid N", which was previously soluble in chloroform/methanol/HCl, is so

TABLE 8

Effect of lipid solvent treatment on rat liver heavy microsomes(untreated or precipitated and washed with TCA)

Microsomal pellets prepared from rat liver were either precipitated and washed twice with 10% TCA, or stored in ice for about 1 hour.

Three sets of each series were then treated as follows:

- A. Analysed without extraction by lipid solvents
- B. Extracted with acetone and the other usual solvents, and the extracts pooled ("1st extract"), then extracted twice with chloroform/methanol/HCl (200:100:1) and these two extracts pooled ("2nd extract ")
- C. Extracted twice with chloroform/methanol/HCl and the extracts pooled ("1st extract"), then extracted with acetone and the other usual solvents ("2nd extract").

The results are the mean of data from two experiments, and are expressed as mg. in the microsomal pellet from 100 gm. liver.

Constituent	Fraction analysed	Previously treated with TCA			Not previously treated with TCA		
		A	B	C	A	B	C
protein (3.25 x non-nucleic acid or non- phospho- lipid N)	Residue	2.73	1.79	1.04	2.65	1.58	1.44
	1st extr. (acet.etc.)	-	0.97	-	-	0.81?	-
	2nd extr. (C/M/HCl)	-	0.03	-	-	0.71?	-
	total extracted	-	1.00	-	-	1.52	-
	or						
	1st extr. (C/M/HCl)	-	-	1.55	-	-	0.64?
	2nd extr. (acet.etc.)	-	-	0.08	-	-	0.47?
	total extracted	-	-	1.63	-	-	1.11
	total recovery	2.73	2.79	2.67	2.65	3.10	2.55
Phospho- lipid	1st extr. (acet.etc.)	-	1.07	-	-	0.98	-
	2nd extr. (C/M/HCl)	-	0.07	-	-	0.10	-
	total extracted	-	1.14	-	-	1.08	-
	or						
	1st extr. (C/M/HCl)	-	-	1.07	-	-	0.97
	2nd extr. (acet.etc.)	-	-	0.04	-	-	0.15
	total extracted	-	-	1.11	-	-	1.12

no longer. These data are based on two experiments in which the TCA-treated specimens gave very similar results.

In the case of the microsomal pellets which were treated with lipid solvents without prior precipitation and washing with TCA, phospholipid was extracted almost completely by the first set of solvents used, just as in the case of the specimens treated with TCA. The behaviour of the "lipid N", however, was slightly different in the two experiments. In both, the total non-phospholipid N extracted by the various lipid solvents from each specimen was about the same as that from the TCA-treated specimens. In one experiment, however, the first solvents (whether the usual series or chloroform/methanol/HCl) extracted little "lipid N", while the later solvents were fully efficient. In the other experiment the greater part of the "lipid N" dissolved in the first set of solvents used, and only a little more in the later solvents. The extraction of "lipid N" by the first series of solvents thus seems to be almost an "all-or-nothing" phenomenon, and the figures in the table marked "?", being averages of the amounts extracted in the two experiments, are misleading.

The finding that, in one experiment, the first set of solvents removed "lipid N" from samples not treated with TCA, seems to contradict those of Table 7, which showed that "lipid N" was not extracted by the usual solvents without prior TCA treatment; the difference may be due to the use of whole homogenate in Table 7, whereas in Table 8 we are dealing with a microsomal preparation. At any rate, the presence of acid does not seem to be necessary for the extraction of "lipid N" by the solvents, since in each

experiment the chloroform/methanol/HCl extraction was no more efficient than extraction with the usual solvents. Nevertheless, the variability observed when acid precipitation was omitted led us to the conclusion that more reproducible "lipid N" extraction could be obtained with prior use of TCA.

The findings obtained from analysis of the N content of the lipid extracts from liver can be summarised as follows:

- (1) The total N extracted by our usual lipid solvents after TCA precipitation represents about 22% of the total acid-precipitable N of the tissue.
- (2) Phospholipid N accounts for only a very small fraction of this N.
- (3) The inclusion of acetone in the extraction scheme does not greatly alter the extent of the loss of N into the lipid solvents.
- (4) The loss of N into ethanol is slightly increased by a prolonged period of extraction.
- (5) Comparison of individual lipid solvents shows that some solvents are rather more effective in removing this N than others, but probably much the same material is removed irrespective of the solvent.
- (6) The material of the lipid solvent extracts is soluble in chloroform/methanol/HCl (200:100:1), and can be fractionated by mixing the chloroform/methanol/HCl solution with 0.2 vols. water. The phospholipid remains in the organic phase, while the other nitrogenous material separates at the interface as a white mat.

- (7) The effect of lipid solvents on whole liver specimens not previously exposed to TCA was examined. The solvents were effective in the removal of phospholipid, but did not remove significant amounts of "lipid N".
- (8) Chloroform/methanol/HCl removes more "lipid N" from TCA-treated microsomes than the other solvents tested, but only if it is used as the initial solvent. It does not remove any further "lipid N" from specimens previously extracted with other solvents.
- (9) Approximately the same amount of "lipid N" could be extracted from microsomal pellets not previously treated with TCA as from those which had been treated. Sometimes the first solvents used removed only phospholipid, the "lipid N" dissolving in the later solvents, but on another occasion both phospholipid and "lipid N" dissolved in the initial series of solvents. This latter result differs from that mentioned in para. (7), possibly because of a difference between microsomes and whole liver. The presence of traces of acid during extraction with lipid solvents does not seem to be necessary for the removal of "lipid N", and the absence of acid is probably not the reason for the findings in para. (7).

The uncertainties associated with the extraction of "lipid N" from whole liver and microsome preparations not previously treated with TCA made us decide in favour of a scheme in which whole liver homogenates or liver cell fractions are precipitated and washed with 10% TCA before

treatment with lipid solvents. For lipid extraction we used the solvents shown in Figure 6 (acetone, ethanol, ethanol/chloroform, ethanol/ether twice and ether) because, although this series removes rather less "lipid N" than does chloroform/methanol, it may for that reason be more selective. Moreover, it allowed us to compare our results with other work carried out here in which this scheme was used.

SECTION II

Chemical nature of the "lipid N"

As has already been explained, about 90% of the nitrogen removed by lipid solvents from acid-precipitated liver residue cannot be accounted for as phospholipid N. We therefore considered in turn the possibilities that it might be (a) nucleic acid N or (b) protein N.

4. Extraction of nucleic acids by lipid solvents:

It has been suggested (Venkataraman and Lowe, 1959) that the treatment of TCA-wet material with ethanol can lead to extraction of up to 30% of the cytoplasmic RNA. While even so great an extraction of RNA would account for only a small fraction of the "lipid N", the claim called for investigation, since procedures involving ethanol extraction of TCA-precipitated material are commonly used in the estimation of nucleic acids in tissues.

In the first instance, we tested the effect of ethanol on liver RNA by carrying out the procedure described by Venkataraman and Lowe. This involved three main stages:

- (a) precipitation and washing with 5% TCA
- (b) extraction of the residue with ethanol (first at 0°, then at room temperature) and with hot ethanol/ether, and
- (c) isolation of the RNA from the washed residue by extraction with sodium chloride solution and re-precipitation by ethanol.

According to Venkataraman and Lowe, omission of stage (b) leads to higher recoveries of RNA in stage (c). We therefore subjected samples of liver cytoplasm to this procedure, omitting and including stage (b), and compared

TABLE 9

Effect of organic solvent extraction
on the RNA content of rat liver cytoplasm

The method is that of Venkataraman and Low (1959). Portions of a 10% homogenate of rat liver in 0.25 M sucrose solution containing 0.18M CaCl_2 were spun for 10 minutes at 600g, and the cytoplasmic supernatants were precipitated and washed 4 times with 5% TCA. Four sets of samples were then treated as follows:

(i) and (ii) were extracted with ethanol and ethanol/ether, and the residue was washed with buffer (stage b).

(iii) and (iv) were washed with buffer without prior extraction by lipid solvents.

(i) and (iii) were then analysed without further treatment.

(ii) and (iv) were extracted with sodium chloride (stage c), and their RNA was estimated after re-precipitation by ethanol.

The results, which are expressed as $\mu\text{g. P}$ in the cytoplasm from 100 mg. liver, were obtained by the Griswold and orcinol methods.

lipid solvent extraction (stage b)	Isolation of RNA (stage c)	P estimation	Orcinol estimation
Extracted by lipid solvents	(i) RNA not isolated	62.0	61.3
	(ii) RNA isolated	59.2	62.1
No lipid solvent extraction	(iii) RNA not isolated	74.0*	52.5*
	(iv) RNA isolated	51.6	58.9

* single sample only

their RNA content. Since any apparent loss of RNA might be due to incomplete recovery in stage (c), we also compared the RNA content of samples in which stage (c) was omitted and the residues analysed directly, with or without extraction of the lipids. The results of these studies, which are given in Table 9, indicate that extraction with ethanol and ethanol/ether in the conditions used does not cause loss of RNA. The loss observed by Venkataraman and Lowe does not appear to be due to incomplete extraction or re-precipitation of RNA in stage (c), since this process seems to be fully efficient.

Secondly, we tested the effect on RNA of our own usual lipid extraction procedure. In Table 10 are shown the results of a comparison of recoveries of RNA and DNA by the Schmidt-Thannhauser procedure, after (a) no extraction of lipids, (b) extraction by all solvents except acetone and (c) extraction by all solvents including acetone. There may be a slight loss of RNA (7-9%) on treatment with the solvents, but it is not nearly so great as is claimed by Venkataraman and Lowe. Moreover, since the estimation of RNA was performed in this case by means of the orcinol reaction, the material removed by the lipid solvents may be orcinol-reacting material other than RNA. Thus even the small loss observed here on treatment with lipid solvents may not be real.

Confirmation that the extraction of RNA, if any, by lipid solvents is very slight, was obtained in another series of experiments where nucleic acid was analysed by the Schneider method of extraction with perchloric acid (PCA) as well as by the Schmidt-Thannhauser procedure. The results, shown in Table 11, indicate that nucleic acids, as estimated by either method, are

TABLE 10

The effect of lipid solvent treatment on the recovery of
nucleic acids from rat liver preparations

Recoveries are given as $\mu\text{g. nucleic acid F per } 100 \text{ mg. liver.}$

The figures in brackets show the difference in RNA recovery relative to the control value.

The results are the mean of those of 4 experiments carried out by the Schmidt-Thannhauser method.

Lipid solvent treatment	RNA	DNA
None	90.5	17.8
Ethanol, etc.	83.9 (-7%)	21.2
Acetone, ethanol, etc.	81.9 (-9%)	21.0

TABLE 11

Effect of lipid solvent extraction on recoveries of nucleic
acids from rat liver samples

The recoveries are given as $\mu\text{g. nucleic acid}$ per 100 mg. liver, and the figures in brackets show the different in recovery of RNA relative to the control value. The results are the mean of those from two experiments. The extraction with FCA was carried out twice with 1.0N FCA for 20 minutes at 70°C.

Lipid solvent treatment	Schmidt-Thannhauser procedure		FCA extraction procedure	
	RNA	DNA	RNA	DNA
None	74.0	17.7	72.7	10.7
Ethanol, etc.	72.8 (~2%)	19.9	70.4 (~3%)	9.9

extracted to a very small extent, or not at all, by the lipid solvents.

The figures obtained for RNA content of the samples by the two methods agree reasonably well, but those for DNA show that the ICA extraction method gives results very much lower than those obtained by the alkaline digestion method of Schmidt and Thannhauser. This and other aspects of nucleic acid estimation were further investigated and are considered in an appendix.

The findings may be summarised as follows:

- i) Either cold 0.7N ICA or 10% TCA may be used as a protein precipitant, and solvent for acid-soluble material, without serious loss of nucleic acids. Contact with cold ICA for 2 hours or longer, however, causes lower recoveries of RNA, although DNA is unaffected; TCA is probably safer as a protein precipitant.
- ii) RNA is efficiently extracted by two 20-minute extractions with 0.5N ICA at 70°, but higher temperatures or concentrations of acid may extract other orcinol-reacting material, and give values which are too high.
- iii) The most favourable conditions for extraction of DNA by ICA are (a) a temperature of 70°C and (b) a ICA concentration between 1 and 3 N. Even so, the extract contains less than 90% of the DNA of the specimen, as measured by the Schmidt-Thannhauser procedure. The latter method is therefore to be preferred for DNA estimation.
- iv) The values obtained by the Ceriotti and orcinol reactions for nucleic acid P in fractions prepared by the Schmidt-Thannhauser method (see Figure 9) agree well with their total P content, if

allowance is made for the "concomitant" P of the supernatant (acid-soluble) fraction.

- v) The most suitable scheme for routine analysis thus seems to be:
- (a) Precipitation and two washings with 10% TCA within 30 minutes.
 - (b) Extraction with lipid solvents as in Fig. 6.
 - (c) Alkaline digestion and fractionation by the Schmidt-Thannhauser method, and measurement of RNA and DNA in the fractions (by estimations and by specific colour reactions).

This scheme was adopted in the experiments on the effect of lipid solvents on nucleic acids, the results of which were given in Tables 10 and 11.

We are now able to account for the discrepancy between the Schmidt-Thannhauser and Schneider procedures in their values for DNA content, and have established the validity of the methods used. The results suggest that lipid solvents remove no DNA and little or no RNA from TCA-precipitated rat liver, so that the "lipid N" removed by the solvents cannot be accounted for, even in part, by nucleic acid N. The further possibility, that it is protein or peptide in nature, will be considered in part B of this section.

In the course of the experiments described in Tables 10 and 11, the lipid-free residue, after digestion in alkali as shown in Figure 9, was separated into (a) an acid-precipitable (DNA) fraction and (b) an acid-soluble (RNA) fraction. During alkaline digestion, some of the protein of the tissue, as well as its RNA, becomes acid-soluble. It was thus possible to estimate the nitrogen content of the whole residue in two parts, and to correct

FIGURE 9

Alkaline digestion method of Schmidt and Thannhauser (1945)

The procedure was applied either to lipid-free powders or to neutralised suspensions of acid-insoluble material which had not undergone extraction with lipid solvents.

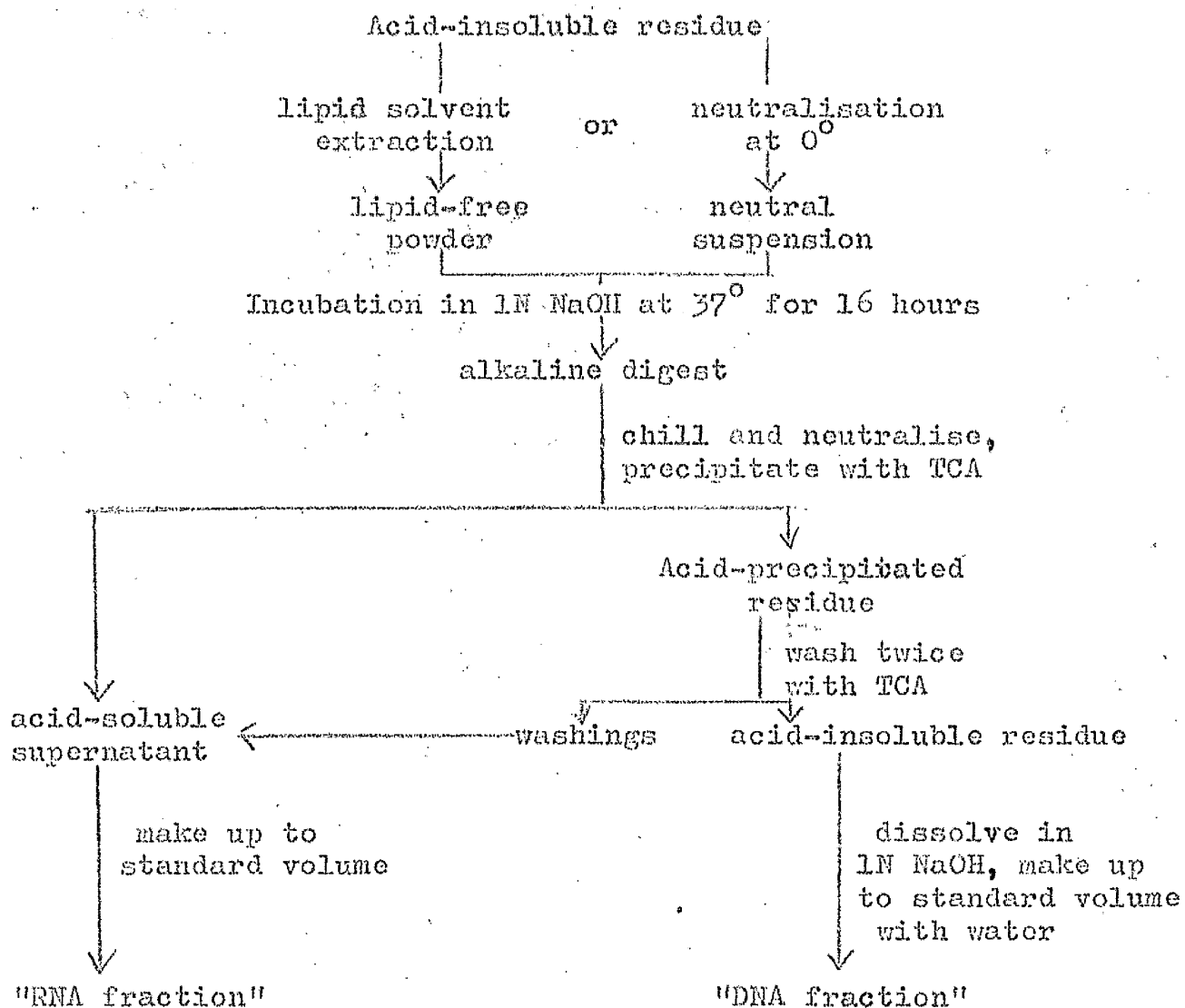


TABLE 12

Effect of lipid solvent extraction on N content of
Schmidt-Thannhauser fractions

The results are expressed as mg. N in the Schmidt-Thannhauser fractions derived from 100 mg. liver.

Treatment	Supernatant	Precipitate	Total
No solvent extraction	1.02	2.02	3.04
After extraction by solvents	0.35	2.01	2.36

the values so obtained for nucleic acid present. The nitrogen loss due to extraction by lipid solvents was consistently about 20-25% of the total non-nucleic acid, non-phospholipid N, and this loss appeared at first to be confined to the supernatant (RNA) fraction obtained on acid precipitation of the Schmidt-Thannhauser digest. In a series of experiments, the acid-precipitable fraction (DNA fraction) did not appear to suffer any loss of N, as may be seen from Table 12.

Later, however, while the total loss of N remained constant at 20-25%, it appeared that the N of the precipitate was diminished as well as that of the supernatant. This difference in distribution was shown not be attributable to the fact that in some of the later experiments mice had been used in place of rats, or to cell fractionation procedures. As was shown later by Fleck and Munro (1961), the Schmidt-Thannhauser procedure is very sensitive, so far as the degradation of protein to acid-soluble products is concerned, to slight variations in the incubation conditions, and no doubt this was the cause of the variability found. The results shown in Table 12, do suggest, however, that the "lipid N" material is more readily degraded by digestion with alkali than the protein of the lipid-free residue.

B. The protein content of the lipid solvent extract:

In the preceding sections it was shown, firstly, that the nitrogen present in the lipid solvent extract from liver is not accounted for by the bases of the phospholipids extracted by these solvents. Secondly, the loss of nucleic acids into these solvents, if it occurs at all, is on such a small scale that it cannot add significantly to the N content of the

lipid solvent extract. Consequently, we must look for other forms of nitrogen in the extract, of which protein is by far the most likely.

The search for protein in the lipid solvents was made in several ways:

(a) The peptide or protein content of the solvent extract was measured by a form of the biuret reaction, and compared with the non-phospholipid N, an estimate of which was obtained by subtraction of the phospholipid N from the total N.

(b) The dried residue from the lipid solvent extract was hydrolysed by mineral acid, and the amino nitrogen content of the hydrolysate measured by the ninhydrin procedure. This figure was compared with that for the non-phospholipid N of the original extract.

(c) The acid hydrolysate was examined for free amino acids by two-dimensional paper chromatography. The amino acid pattern which appeared under these conditions was compared with that of a chromatogram of a similar specimen not subjected to hydrolysis.

(d) Paper electrophoresis was carried out in an attempt to separate the constituents of the lipid solvent extracts, and specific stains were used to identify protein and lipid material.

(e) An attempt was made to separate protein material by passage through a column of DEAE cellulose.

(a) Comparison of the value by biuret reaction with the "lipid N" content of the extract:

There are many modifications of the biuret reaction for polypeptides

and proteins, some of which, like the Lowry method, include reagents which react also with the phenolic amino acids. Since we were uncertain of the amino acid composition of our material, we chose to use the original biuret reaction, in the quantitative modification described by Cornall et al (1949) for the measurement of plasma protein. Since we had no isolated protein from the lipid extract to use as a standard in the preparation of a calibration curve, and since it is known that, at least with the Lowry procedure, the colour developed by different proteins is quite variable, we used two standard proteins to calibrate our test. One was egg albumin, the other casein. Both in fact gave similar curves by the quantitative biuret reaction.

On the basis of these standards, the "protein" content of the lipid extract from rat liver was found to agree well with the "lipid N" content, if the protein is assumed to contain 16% of N. The estimates obtained are given in Table 13.

This result was exploited in later experiments, such as those on radioactive incorporation (see Section VI), in which we required a rapid and simple method of estimating comparative amounts of "protein" in lipid solvent extracts. We used the modification of the biuret reaction described by Lowry et al (1951), and compared the values for "lipid N" so obtained with those based on N analysis by the micro-Kjeldahl method. In a series of 42 samples the Lowry values were, on average, 8% higher than the micro-Kjeldahl (if we assume that the N content of the "protein" is 16%).

(b) Comparison of the value by the ninhydrin reaction with the "lipid N" content of the extract:

The colorimetric ninhydrin reaction of Yemm and Cocking (1955) was

TABLE 13

Estimates of "lipid N"

This table shows the comparative results of various estimations carried out on a pooled lipid extract of rat liver. The results for extracts including and omitting acetone have been combined. Each estimation was carried out on duplicate samples obtained from one animal. The figures are calculated on the assumption that the protein estimated by the ninhydrin, biuret and chromatography methods has a nitrogen content of 16%.

In this experiment, extraction by lipid solvents had reduced the nitrogen content of the acid-insoluble residue by 522 $\mu\text{g.}$ per 100 mg. liver, of which 482 $\mu\text{g.}$ were non-phospholipid N.

Method of estimation	$\mu\text{g. N per 100 mg. liver}$
Loss* through solvent treatment	482
Lipid extract:	
Nessler N*	482
biuret (quantitative)	450
ninhydrin (quantitative)	443
paper chromatography (approx.)	450

* corrected for phospholipid N

selected, since it has been found reliable in other connections in this laboratory. Again, two proteins (egg albumin and casein) were hydrolysed with mineral acid to yield free amino acids, and used as standards. Very similar results were obtained from the two standards in relation to their nitrogen content. The same procedure was then applied to an acid hydrolysate of the lipid extract, which had been evaporated to dryness. The free amino acid content given by the ninhydrin reaction showed good agreement with the "lipid N" measured by the Nessler method and corrected for phospholipid N, as shown in Table 13. It also agrees with the results of the biuret reaction, referred to above.

(c) Two-dimensional paper chromatography of the hydrolysed lipid extract:

The mineral acid hydrolysate of the dried lipid extract was also subjected to two-dimensional paper chromatography for the separation of amino acids liberated by hydrolysis. As a control procedure, an equivalent amount of the same lipid extract, not subjected to hydrolysis, was similarly treated and examined by ninhydrin staining for the presence of free amino acids or peptides.

This procedure was applied to three kinds of lipid extract of liver:

- (i) Pooled extract obtained by the use of all the usual lipid solvents, including acetone.
- (ii) Pooled extract obtained by the use of all the solvents with the exception of acetone.
- (iii) An extract made with chloroform/methanol/HCl (200:100:1).

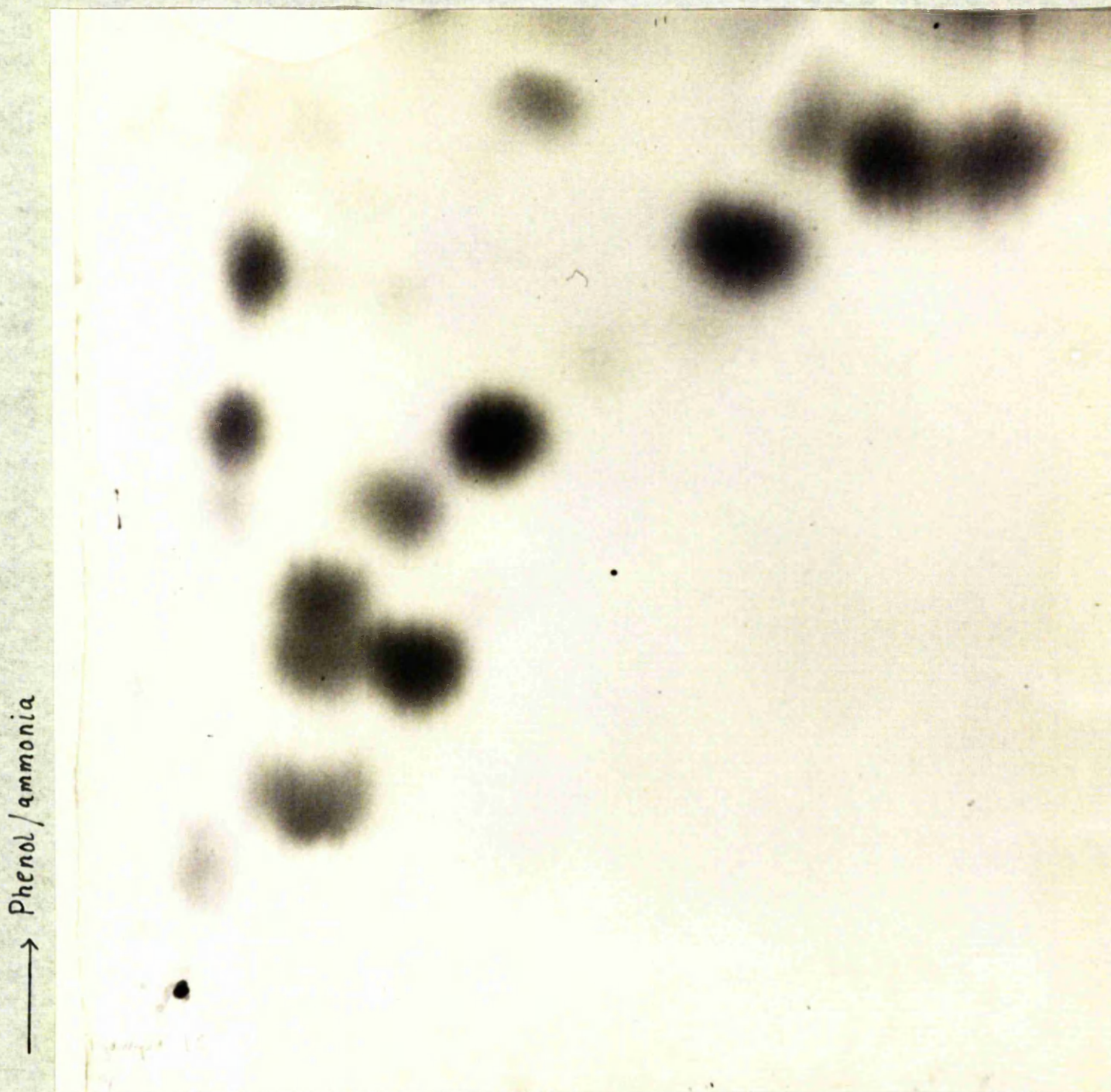
In all cases, the liver specimens had previously been treated with 10% TCA.

Fig. 10 shows the pattern obtained with a hydrolysate of pooled extract (i) (all solvents), and Fig. 11 the pattern from the corresponding unhydrolysed extract. The amino acid "map" is typical of a protein, and a rough estimate by inspection of the quantity of protein on the chromatogram (by Dr. G. Leaf) agreed approximately with the amount of nitrogenous material applied (Table 13). Moreover, the unhydrolysed sample shows no free amino acids, its only ninhydrin-positive material being a small, faint spot, stationary in butanol/acetic acid and moving just behind the solvent front in phenol/ammonia, probably consisting of some small peptides. Thus the nitrogenous material of the lipid extract seems to be mainly polypeptide in nature.

The hydrolysates of the extract prepared without the use of acetone (ii) and of the chloroform/methanol/HCl extract (iii) yielded chromatograms (Figs. 12 and 13 respectively) very similar to that obtained from the first extract (i), both in pattern and amount. This finding lends support to the theory that the different solvents remove the same material. Again, the chromatograms of the unhydrolysed extracts showed only the single spot described above, as may be seen in the case of the chloroform/methanol/HCl extract in Fig. 14.

It is, of course, possible that the unhydrolysed material applied to the chromatograms shown in Figs. 11 and 14 did not all remain at the origin, and that, although not capable of reacting with ninhydrin, it nevertheless moved on the paper. The unhydrolysed extract, for instance, might contain some peptides too large to give a strong colour with ninhydrin but still

FIGURE 10



————→ Butanol /acetic acid

Paper chromatogram of hydrolysate of lipid solvent extract of
TCA-precipitated residue of whole liver. The solvents used were
those shown in Figure 6.

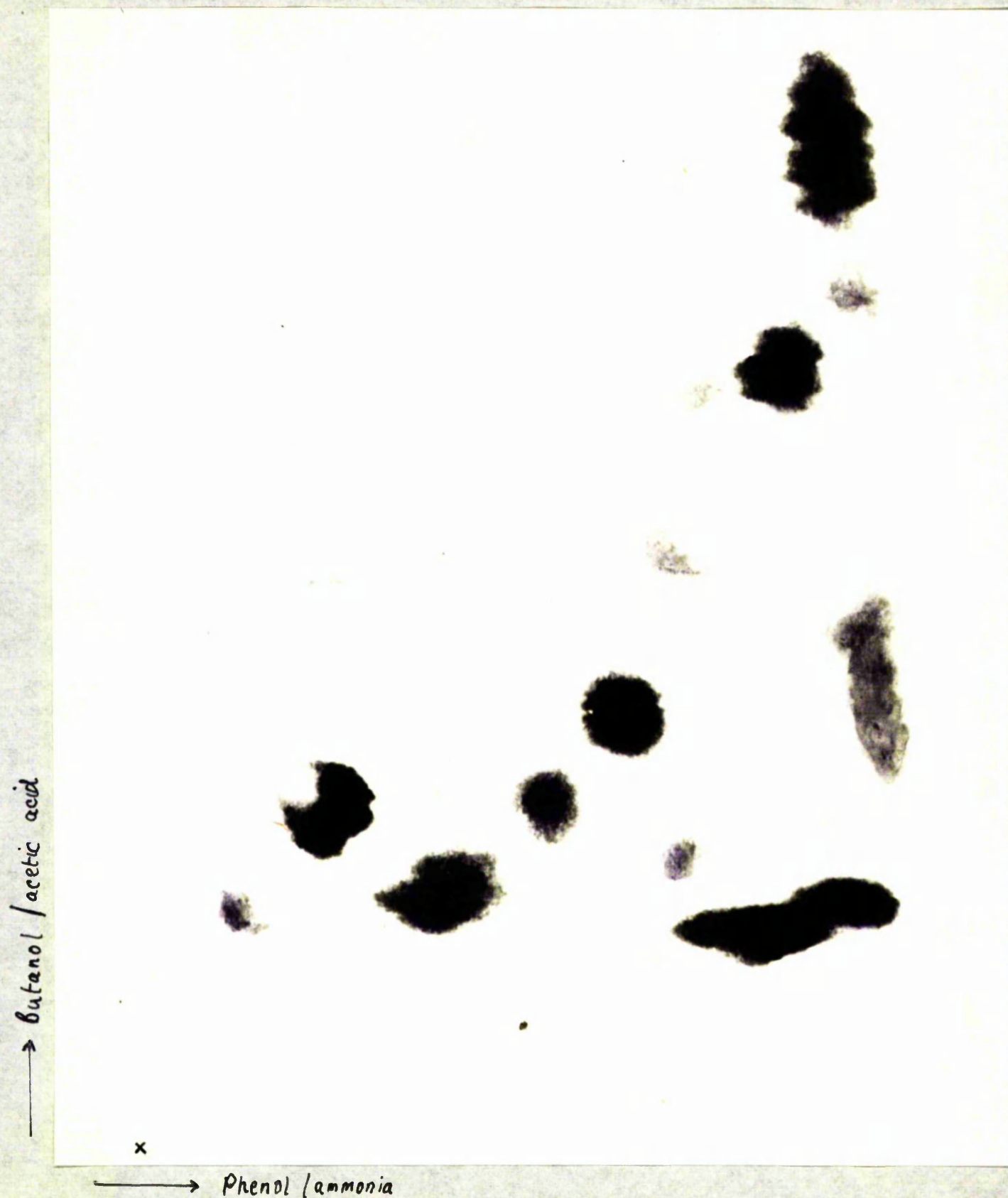
FIGURE 11



Butanol / acetic acid ←

Paper chromatogram of unhydrolysed lipid solvent extract of
TCA-precipitated residue of whole liver. The solvents used
were those shown in Figure 6. Compare with Figure 10.

FIGURE 12



Paper chromatogram of hydrolysate of lipid solvent extract of TCA-precipitated residue of whole liver. The solvents used were those shown in Figure 6, with the omission of acetone.

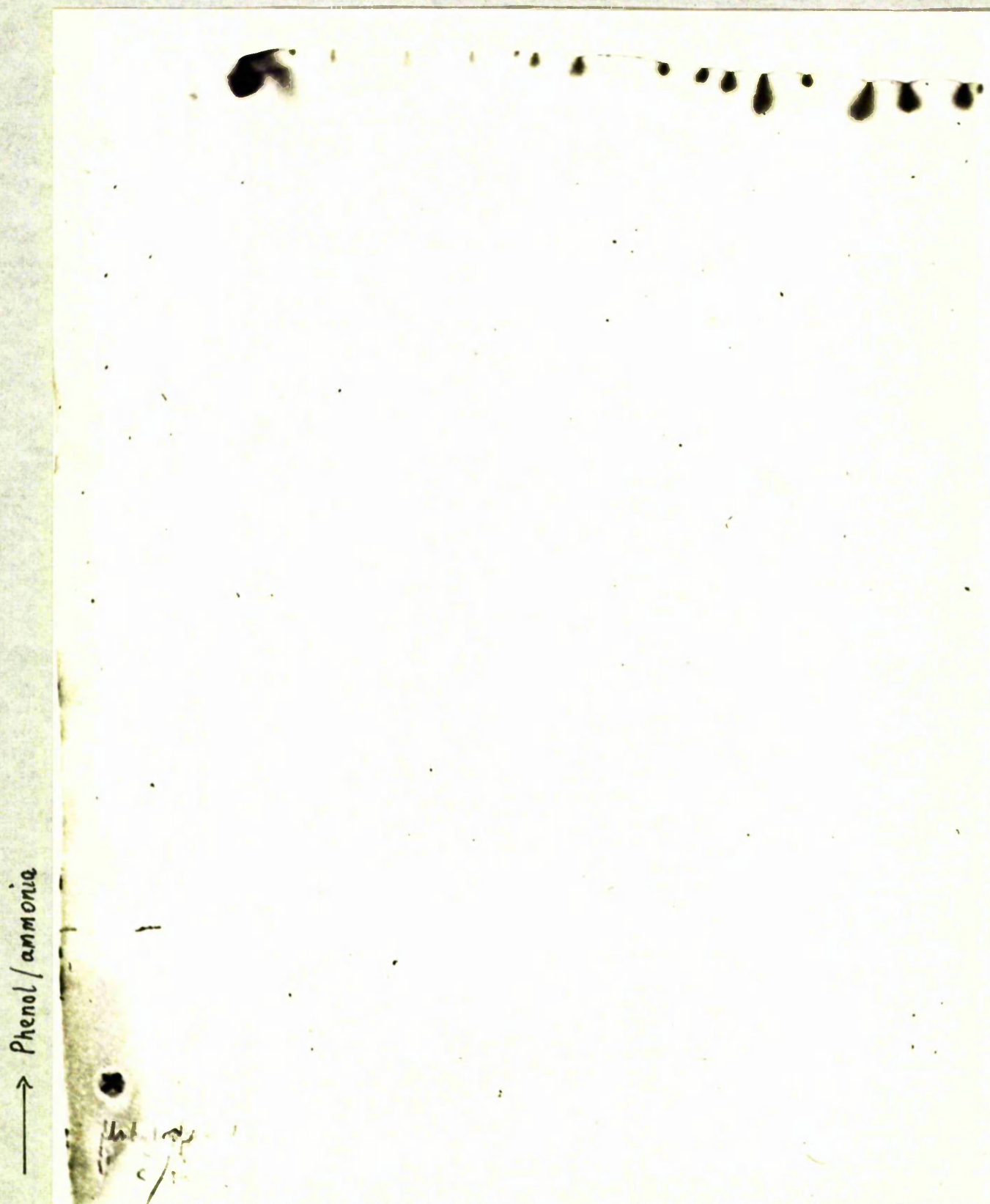
FIGURE 13



————→ Butanol / acetic acid

Paper chromatogram of hydrolysate of a chloroform/methanol/HCl
extract of the TCA-precipitated residue of whole liver.

FIGURE 14



————→ Butanol/acetic acid

Paper chromatogram of unhydrolysed chloroform/methanol extract of TCA-precipitated residue of whole liver. Compare with Figure 13.

mobile in the solvents used. If so, it should be possible to separate them by cutting up an unstained chromatogram after one-dimensional chromatography of the unhydrolysed extract. The position of any such peptides could then be determined by eluting the strips, hydrolysing the eluates with acid and staining for free amino acids with ninhydrin. The process could be repeated with a second solvent, if further separation were needed.

Attempts to perform such a separation, however, were unsuccessful; each of the hydrolysed eluates from the first chromatogram was run on a second strip, which was then stained with ninhydrin for free amino acids, but all the strips were blank. It was not clear whether this was due to inefficient elution of the first strip or simply to insufficiency of material. Even if all the unhydrolysed material remained at the origin of the first chromatogram, this could not account for the failure to recover it, since the whole of the first strip, including the origin, was eluted.

(d) Paper electrophoresis of the lipid solvent extract:

Electrophoresis was carried out on extracts prepared with a single solvent, acetone in one case and ethanol/chloroform in the other, each solvent being the first used after TCA. The extracts were prepared from the soluble fraction of rat liver microsomes treated with sodium deoxycholate, a fraction which, as will be shown later, is particularly rich in "lipid N".

After electrophoresis had been carried out, and the proteins coagulated by heat, the paper strip was split lengthwise. One half was stained for protein with bromophenol blue, the other for lipid with Sudan Black.

It was clear that almost all the material applied had remained at the origin, where it stained strongly with both bromophenol blue and Sudan Black. This immobility may have been due to denaturation of the protein by the treatment with TCA and organic solvents. The strips from both extracts, however, also showed another band, positive to bromophenol blue and to Sudan Black, which moved rapidly towards the anode, e.g. the band from the acetone extract usually moved about 15 cm. and that from the ethanol/chloroform extract about 11 cm. An extract prepared with ethanol as the only solvent gave a similar, but much slower-moving, band (generally about 3 - 5 cm. from the origin). In all cases the position of the bromophenol blue-positive band corresponded to that of the Sudan Black-positive band, and the different extracts gave bands of roughly equal intensity. It is probable that the material is the same in each case, and that the differences in mobility are due simply to the effect of the various solvents used in the extractions. It is possible also that the band consists of albumin, which is known to be soluble in ethanol after precipitation with TCA (Levine, 1954) without being denatured by such treatment. Albumin does move fairly rapidly during electrophoresis, and is capable of binding lipids.

Attempts to separate the protein material of the lipid solvent extract by means of paper electrophoresis were thus unsuccessful, either because the protein had been so modified during extraction that it was no longer mobile, or because the conditions employed were unsuitable for this particular system. Whatever may have been the material responsible for the faint band that was found, the great bulk of the extract remained at the point of

application.

(e) Separation of the protein of the lipid extract by means of a column of DEAE cellulose:

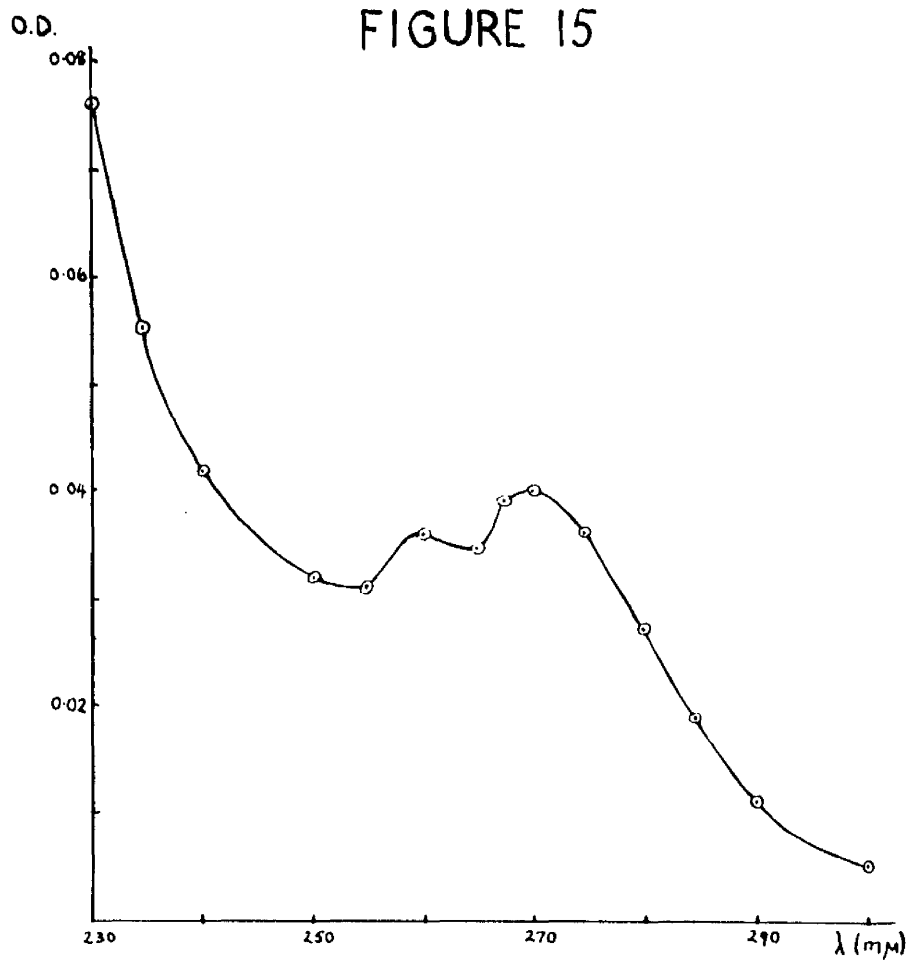
Further attempts were made to separate the components of an acetone and an ethanol/chloroform extract of the deoxycholate-soluble fraction of rat liver microsomes (the same extracts as were used for paper electrophoresis) by passage through a column of DEAE cellulose and elution by a range of phosphate buffers of varying pH and ionic strength. Owing to the technical difficulty of applying a solution of protein in an organic solvent to an aqueous column, the material of the extract was not well adsorbed on the column; a brown band was seen to move a little way down the column immediately, and then to remain in the same position throughout the elution. Probably the lipid material clogged the cellulose.

The column was eluted with a range of phosphate buffers of increasing ionic strength over the pH range 7.6 - 4.1, and the emerging fractions were tested for protein by the Lowry reaction, which had been shown to react satisfactorily with this material. The eluting buffers were changed in a stepwise fashion, and after the introduction of each new eluent the fractions contained small quantities of Lowry-positive material, but no clear peaks were obtained, and most of the protein stayed on the column.

Possibly some of the material might have been eluted by more alkaline buffers.

The ultra-violet absorption spectrum of one of the Lowry-positive eluates (from the acetone extract) is shown in Fig. 15. Since the amount of material

FIGURE 15



UV absorption spectrum of purified
acetone extract of TCA-precipitated
rat liver microsomes

present is so small, the optical densities are not high enough for the spectrum to be regarded as very accurate. It does, however, show a small peak at about 269 m μ , the significance of which is not clear. It is a little too far to the right for ribonucleic acid, which generally shows an absorption maximum at 260 m μ , and slightly to the left of the maximum (275 m μ) of protein. Similar spectra were obtained from other fractions, but in all cases the actual readings were very low.

Although it was found earlier that the lipid solvents did not extract significant quantities of nucleic acid, very little would be needed to give the absorption seen in Fig. 1b.

The nature of the "lipid N" - summary

Our findings may be summarised as follows:

1. Only a very small fraction of the "lipid N" is accounted for as phospholipid N (section I).
2. Little if any of the "lipid N" consists of RNA, and it contains no DNA (section IIA).
3. An approximate estimate by a quantitative biuret method showed that most of the non-phospholipid N of an unhydrolysed lipid solvent extract was in the form of peptide linkage (section IIB (a)).
4. By means of a quantitative ninhydrin reaction it was shown that most of the N in a hydrolysate of solvent extract was amino-N (section IIB (b)).
5. Ninhydrin staining of paper chromatograms of hydrolysed extracts indicated that most of the "lipid N" could be accounted for as amino acids. The picture was that of a typical protein hydrolysate. Very little ninhydrin-positive material could be seen in the chromatograms of unhydrolysed extracts. Thus the amino acids of the solvent extracts are not free, but are bound in a linkage which is stable to the conditions of chromatography, and which prevents reaction with ninhydrin. Attempts to separate any proteins or peptides by paper chromatography were unsuccessful (section IIB (c)).
6. By the use of specific stains for protein and lipid after paper electrophoresis, it was shown that the bulk of the protein and lipid material of the solvent extract did not move in the conditions used for electrophoresis. The material at the origin stained strongly for both lipid and protein, as did a faint band which moved fairly rapidly towards the anode (section IIB (d)).

7. It was found difficult to elute the extract material from a column of DEAE cellulose, and attempts to separate its proteins by this means were not successful (section IIB (e)).

SECTION III

Subcellular distribution of the "lipid N"

In the preceding sections it was shown that a protein-like material is removed by the action of the organic solvents used to extract lipids from the TCA-precipitated residue of rat liver.

We next investigated the abundance of this material relative to that of other cell constituents, and its subcellular distribution in different fractions of the cell. The content of lipid protein and other cell components was analysed in both rat and mouse liver, and Table 14 shows the results. These demonstrate that the distribution in the two species is essentially similar. In the rat, "lipid protein" accounts for 22% of the total dry weight and 24% of the protein, and in the mouse for 27% of the total dry weight and 33% of the protein. Thus lipid protein must be considered as a major component of the cell, greater in amount than phospholipid.

The next question studied was whether the "lipid protein" was evenly distributed throughout the cell in proportion to the protein content of each fraction, or whether it was especially abundant in any of these fractions. Homogenates of both rat and mouse liver were prepared in sucrose solution, and portions were fractionated according to the scheme shown in Figure 5 to yield mitochondria, microsomes (heavy and light) and cell sap. The four fractions from each sample were precipitated and washed with TCA, and extracted with lipid solvents in the same way as the samples of whole homogenate described earlier. The rats had all been maintained on a diet containing

TABLE 14

Composition of whole cell of rat and mouse liver

The protein figures are calculated as "lipid N" x 6.25 and (total N of lipid-free residue - nucleic acid N) x 6.25 respectively. The "total dry weight" represents simply the total weight of the constituents estimated, and other constituents of the liver (e.g. the acid-soluble material) are not included. Each figure is the mean of the results of an experiment in which the livers of two animals were analysed separately.

	Rat		Mouse	
	mg. per 100 mg. liver	% of total dry weight	mg. per 100 mg. liver	% of total dry weight
Lipid protein	4.57	22.2	6.09	26.6
Non-lipid protein	12.00	58.2	12.06	52.8
Phospholipid	2.95	14.4	3.25	14.2
RNA	0.75	3.65	1.18	5.18
DNA	0.25	1.22	0.25	1.10

an adequate amount of protein, and had been fasted for 18 hours before the experiment. The mice had been maintained on a stock diet, and were not fasted beforehand.

Tables 15 and 16 give the composition of the cytoplasmic fractions obtained from these livers. All cell fractions are seen to contain some "lipid N", but the distribution is not uniform. The membranous fractions, i.e. the mitochondria and the heavy microsomes, are much richer in this component than the light microsomes. This pattern suggests that the "lipid N" may be in some way connected with the membranes or endoplasmic reticulum of the cell. In general, the subcellular distribution of "lipid N" follows that of the phospholipid. An exception may be seen in the case of the cell sap of rat liver from animals fasted overnight, which has a high content of "lipid N". It is possible that this represents fragments of the endoplasmic reticulum, which breaks down during fasting. Some support for this hypothesis may be obtained from the data given later in Table 20, which shows the "lipid N" content of the microsomes and cell sap of animals fasted for 18 hours, and killed either fasting or 1½ hours after a protein meal. The "lipid N" content of the microsomes is lower in the fasted animals than in those fed protein, and that of the cell sap is correspondingly higher.

The high content of lipid protein in the mitochondria and heavy microsomes may be seen more clearly in Table 17, which is based on the data of Tables 15 and 16. It shows the lipid protein content of the cell fractions of rat and mouse liver expressed as a percentage of the total protein in each

TABLE 15

Composition of cell fraction of rat liver

The animals were maintained on a diet containing adequate protein and were fasted for 18 hours before being killed. The lipid-free protein was calculated as (N of lipid-extracted residue - nucleic acid N) x 6.25. The results are expressed as $\frac{100 \text{ g.}}{\text{actual body wt.}}$ mg./liver/100 g. body weight of each constituent in the cell fraction from one whole liver), and also as a percentage of the total weight of the constituents estimated in each cell fraction.

Results	Cell fraction	"lipid N" x 6.25	Lipid-free protein	phospho- lipid	RNA
mg./liver/ 100 g. body weight	Mitochondria	28.3	65.3	18.0	2.33
	Heavy microsomes	36.7	48.0	35.2	8.48
	Light microsomes	8.7	35.6	9.4	5.71
	Cell sap	35.6	104	2.6	2.13
% of total dry weight of cell fraction	Mitochondria	24.9%	57.6%	15.8%	2.05%
	Heavy microsomes	28.5%	37.3%	27.3%	6.60%
	Light microsomes	14.6%	60.0%	15.8%	9.65%
	Cell sap	24.5%	72.0%	1.8%	1.47%

TABLE 16

Composition of cell fractions of mouse liver

The mice were maintained on a stock diet and were not fasted before being killed. The non-lipid protein was calculated as $(N \text{ of lipid-free residue} - \text{nucleic acid N}) \times 6.25$. The results are expressed as mg, of each constituent in the cell fraction derived from 100 mg. liver, and as a percentage of the total weight of the constituents estimated in each fraction.

Results	Cell fraction	"Lipid N" x 6.25	Lipid-free protein	Phospho- lipid	RNA*
mg. per 100 mg. liver	Mitochondria	0.79	1.85	0.64	0.26
	Heavy microsomes	0.65	2.15	1.22	0.49
	Light microsomes	0.15	1.02	0.34	0.29
	Cell sap	0.34	4.20	0.18	0.11
percent. of cell fraction	Mitochondria	22.3%	52.3%	18.1%	7.3%
	Heavy microsomes	14.4%	47.6%	27.0%	10.9%
	Light microsomes	8.3%	56.8%	18.9%	16.0%
	Cell sap	7.0%	87.0%	3.7%	2.3%

TABLE 17

The lipid protein content of cell fractions of rat and mouse liver

The figures given in this table are calculated from the data of Tables 15 and 16. The lipid protein is calculated as (non-phospholipid N of the lipid solvent extract x 6.25), and is expressed

- (a) as mg. lipid protein in the cell fraction from the whole liver x $\frac{100 \text{ g.}}{\text{actual body weight}}$ in the case of the rat, or as mg. lipid protein in the cell fraction derived from 100 mg. liver in the case of the mouse, and
- (b) as a percentage of the total protein content of the cell fraction.

Cell fraction	Rat		Mouse	
	Weight	Percentage	Weight	Percentage
Mitochondria	28.3)	30.3%	0.79)	30.0%
Heavy microsomes	36.7)	73.7	0.65)	1.59
Light microsomes	8.7)	19.7%	0.15)	12.8%
Cell sap	35.6	25.5%	0.34	7.2%

fraction. This table also shows clearly that the greater part of the lipid protein is contained in the particulate fractions of the cell - about two-thirds of the total lipid protein of the cell in the case of the rat, and five-sixths in the case of the mouse.

SECTION IV

The effect of variation in diet on the "lipid N" content of rat liver

As we have just seen, the membranous structures of the liver cell (the mitochondria and heavy microsomes) appear to contain the greater part of the "lipid N" of the cell (Table 17). This finding suggests a possible connection with the endoplasmic reticulum.

It has been shown (Fawcett, 1955) that the amount of endoplasmic reticulum in the liver cell varies with the dietary state of the animal. Starvation for 6 days leads to loss of the reticulum, which regenerates rapidly after a protein (but not carbohydrate) meal. If, therefore, our "lipid protein" forms part of the endoplasmic reticulum, it may be expected to vary with diet. Critical factors in the diet might be protein level and calorie intake. Animals were therefore maintained for 11 days on diets deficient in protein (LP) or containing an adequate amount of protein (HP). Each group was subdivided into three sets, receiving either no energy supplement, a supplement of carbohydrate or a calorifically equivalent supplement of fat, and all the animals were fasted for 18 hours before they were killed. Table 18 shows the composition of whole-cell homogenates of liver from these rats. Although there is less "lipid N" in all three groups of "LP diet" than in the corresponding "HP diet" groups, there is also a fall in the lipid-insoluble protein fraction, so that the percentage of the total protein which is extracted by lipid solvents is not decreased by protein deficiency. Only in the groups which lacked any energy supplement (and which were in fact deficient in calories) is there any evidence of

TABLE 18

Composition of rat liver (whole cell) on various diets

The dietary groups were as described in the text: each consisted of three animals. The protein content of the liver was calculated as in Table 14, and the results are expressed as

(a) mg. constituent in one whole liver $\times \frac{100 \text{ g.}}{\text{actual body weight}}$ and

(b) % of the total weight of the liver constituents estimated.

The figures in brackets represent the lipid protein as a percentage of the total protein.

Results	Diet and supplement	"lipid N" $\times 6.25$	lipid-free protein	phospho- lipid	RNA	DNA
mg. per liver per 100 g. body wt.	HP low energy	117 (23%)	405	67.5	22.3	8.9
	HP + carb.	103 (17%)	526	82.5	29.7	10.3
	HP + fat	126 (19%)	580	96.0	31.7	10.3
	LP low energy	77 (18%)	360	55.0	17.3	8.4
	LP + carb.	90 (18%)	394	65.5	24.5	9.3
	LP + fat	102 (18%)	466	68.0	25.7	10.4
% of total weight	HP low energy	18.8%	65.3%	10.9%	3.6%	1.43%
	HP + carb.	13.7%	70.0%	10.8%	3.95%	1.37%
	HP + fat	14.9%	68.7%	11.3%	3.75%	1.22%
	LP low energy	14.9%	69.8%	10.6%	3.35%	1.63%
	LP + carb.	15.4%	67.4%	11.3%	4.2%	1.59%
	LP + fat	15.2%	69.4%	10.1%	3.8%	1.55%

preferential loss of "lipid protein".

It is, of course, possible that variations in diet affect the "lipid N" of only some fractions of the cell, such as perhaps the microsomes. If so, the effect might be obscured by the presence of other fractions in whole cell homogenates. More detailed analysis (Tables 19(a) and 19(b)) of the protein and other constituents of the four main cytoplasmic fractions of the liver cell, however, reveals little variation with diet in the "lipid N" content of the microsomes or any other fraction. There seems to be no difference in the composition of the fractions with different levels of protein or with different calorie intakes, although the total weight is reduced in protein deficiency, and may be slightly increased by the addition of a fat supplement to the diet. In particular, the amount of "lipid N" in the large particles (mitochondria and "heavy microsomes"), relative to that in the "light microsomes" and cell sap, does not show very much variation with diet. It may therefore be concluded that dietary variations of the type examined here can cause changes in the total amounts of cell fractions without affecting their intrinsic composition.

Specimens of liver from two of the dietary groups were examined in the electron microscope, so that differences in the analytical data might be related to differences in the appearance of the cell. The liver of a rat maintained on the high protein diet with a carbohydrate supplement (Figure 16) shows a little more reticulum than that of a rat maintained on the diet deficient in protein, with the same supplement (Figure 17). The latter also shows the prominent nucleolus characteristic of protein deficiency.

TABLE 19a

The composition of cell fractions of liver from rats

maintained on different diets

Each dietary group consisted of two rats. Their livers were analysed separately, and the results are expressed as

mg. constituent in one whole liver $\times \frac{100 \text{ g.}}{\text{actual body weight}}$

Cell fraction	Diet	Lipid protein*	Non-lipid protein	Phospho-lipid	RNA
Mitochondria	HP LE	26.4	55.4	15.4	1.81
	HP + C	23.1	60.5	16.6	2.30
	HP + F	35.5	80.1	21.9	2.88
	LP LE	20.2	51.8	13.0	1.50
	LP + C	14.5	46.6	13.5	2.20
	LP + F	24.7	61.3	21.0	3.30
Heavy microsomes	HP LE	37.8	42.5	31.2	6.55
	HP + C	35.7	37.6	35.8	7.71
	HP + F	36.7	64.0	38.5	11.2
	LP LE	31.3	42.2	28.2	6.01
	LP + C	27.6	45.3	28.2	8.38
	LP + F	32.5	46.3	30.6	9.00
Light microsomes	HP LE	9.7	35.8	9.7	5.33
	HP + C	5.2	30.6	8.0	5.20
	HP + F	11.1	40.5	10.5	6.60
	LP LE	6.9	31.5	8.2	4.55
	LP + C	7.0	26.6	6.7	4.10
	LP + F	6.3	33.6	8.3	5.29
Cell sap	HP LE	35.8	102	2.2	2.05
	HP + C	36.2	84	2.7	1.90
	HP + F	34.9	125	3.0	2.45
	LP LE	21.4	85	2.0	1.58
	LP + C	25.0	93	2.8	2.03
	LP + F	36.7	115	3.35	2.53

* Lipid protein = "lipid N" \times 6.25

C - carbohydrate supplement

LE - low energy (no supplement)

F - fat supplement

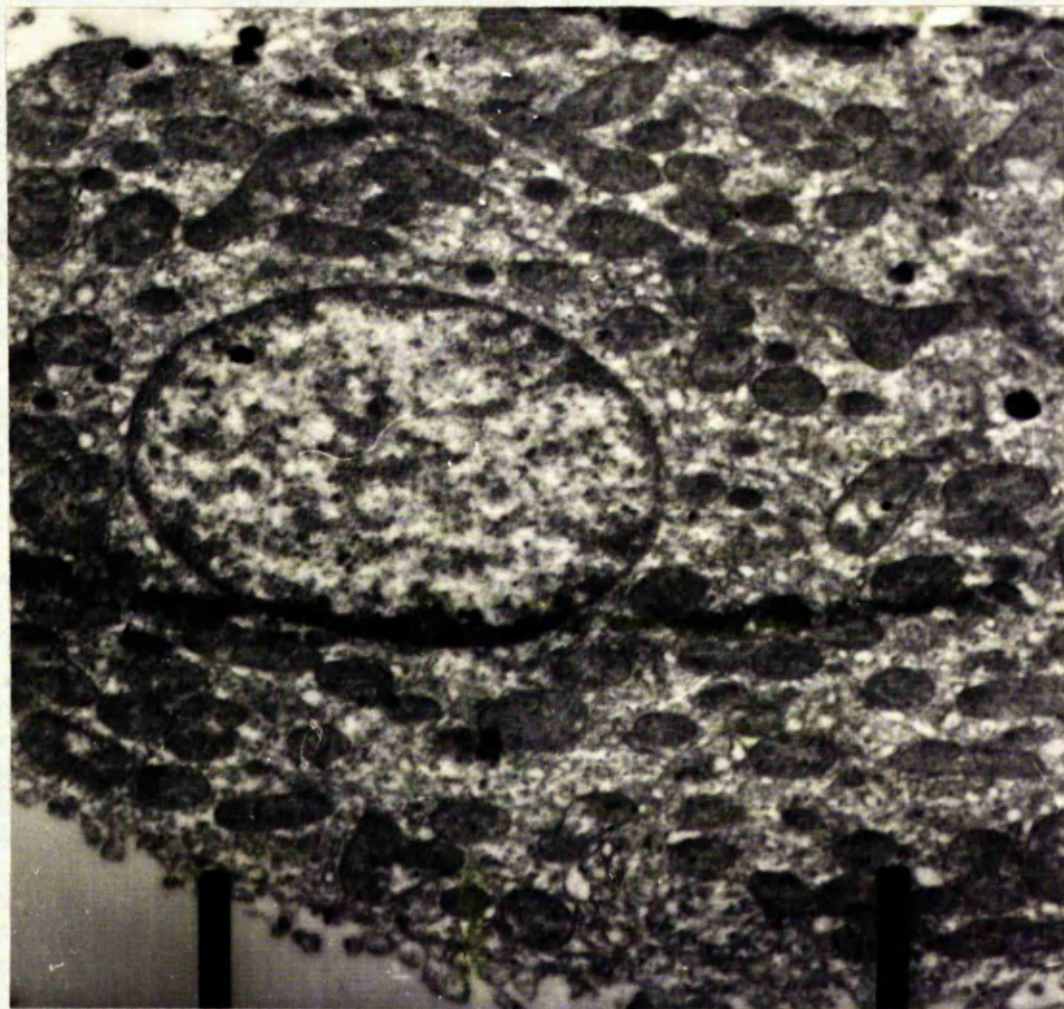
TABLE 19b

Percentage composition of cell fractions of liver from rats
maintained on different diets

The results are those of Table 19a, expressed as a percentage of the total weight of each cell fraction. (The "total weight" is taken to be the sum of the weights of the constituents estimated.) The lipid protein is also expressed as a percentage of the total protein in each cell fraction.

Cell fraction	Diet	Lipid protein		Non-lipid protein	Phospho-lipid	RNA
		% of tot.	% of prot.			
Mitochondria	HP LE	26.9	33.5	56.5	16.0	1.83
	HP + C	22.6	28	59.0	16.4	2.23
	HP + F	25.3	31	57.2	15.6	2.05
	LP LE	23.4	32	60.0	15.0	1.65
	LP + C	18.9	28.5	60.8	17.6	2.86
	LP + F	22.3	28.5	55.7	19.0	2.99
Heavy microsomes	HP LE	32.2	47.5	36.0	26.4	5.71
	HP + C	30.5	49	32.2	26.8	7.67
	HP + F	24.4	37	42.5	25.5	7.45
	LP LE	29.2	42	39.4	25.5	5.60
	LP + C	25.2	38	41.3	25.8	7.65
	LP + F	27.3	42	39.1	25.8	7.60
Light microsomes	HP LE	16.0	21	59.9	16.1	8.87
	HP + C	10.6	11	62.5	16.3	10.6
	HP + F	16.2	22	59.0	15.4	9.60
	LP LE	13.3	18	61.4	16.1	8.87
	LP + C	15.8	20	60.0	15.1	9.22
	LP + F	11.8	17	63.0	15.6	9.95
Cell sap	HP LE	25.3	26.5	72.0	1.56	1.42
	HP + C	29.1	30	67.1	2.16	1.53
	HP + F	24.1	23	75.5	1.81	1.48
	LP LE	19.4	20	77.3	1.82	1.43
	LP + C	20.3	23	76.0	2.28	1.58
	LP + F	23.3	24.5	73.1	2.12	1.60

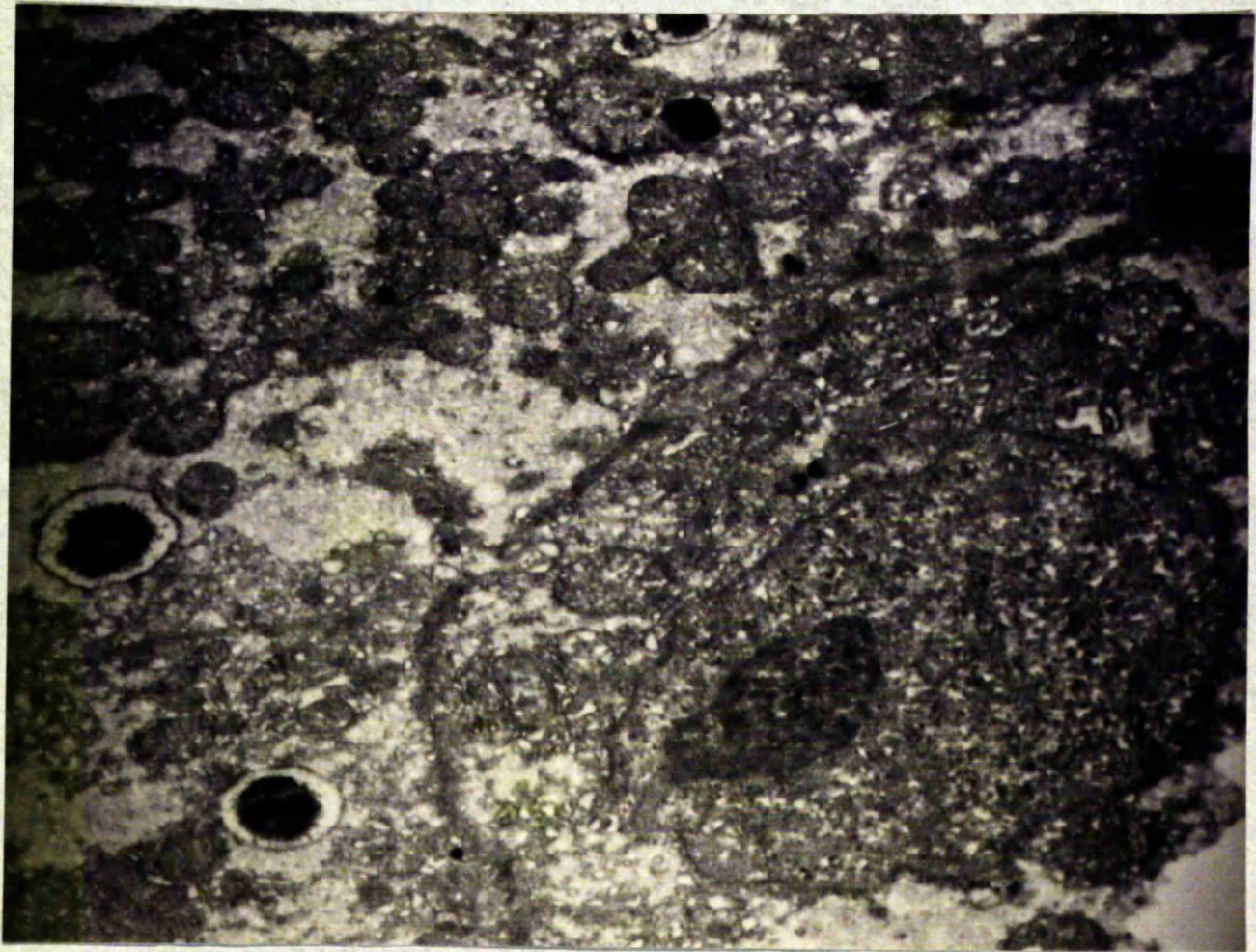
FIGURE 16



Magnification x 10,200

Electron micrograph of liver tissue from rat maintained on diet containing adequate protein, and fasted overnight.

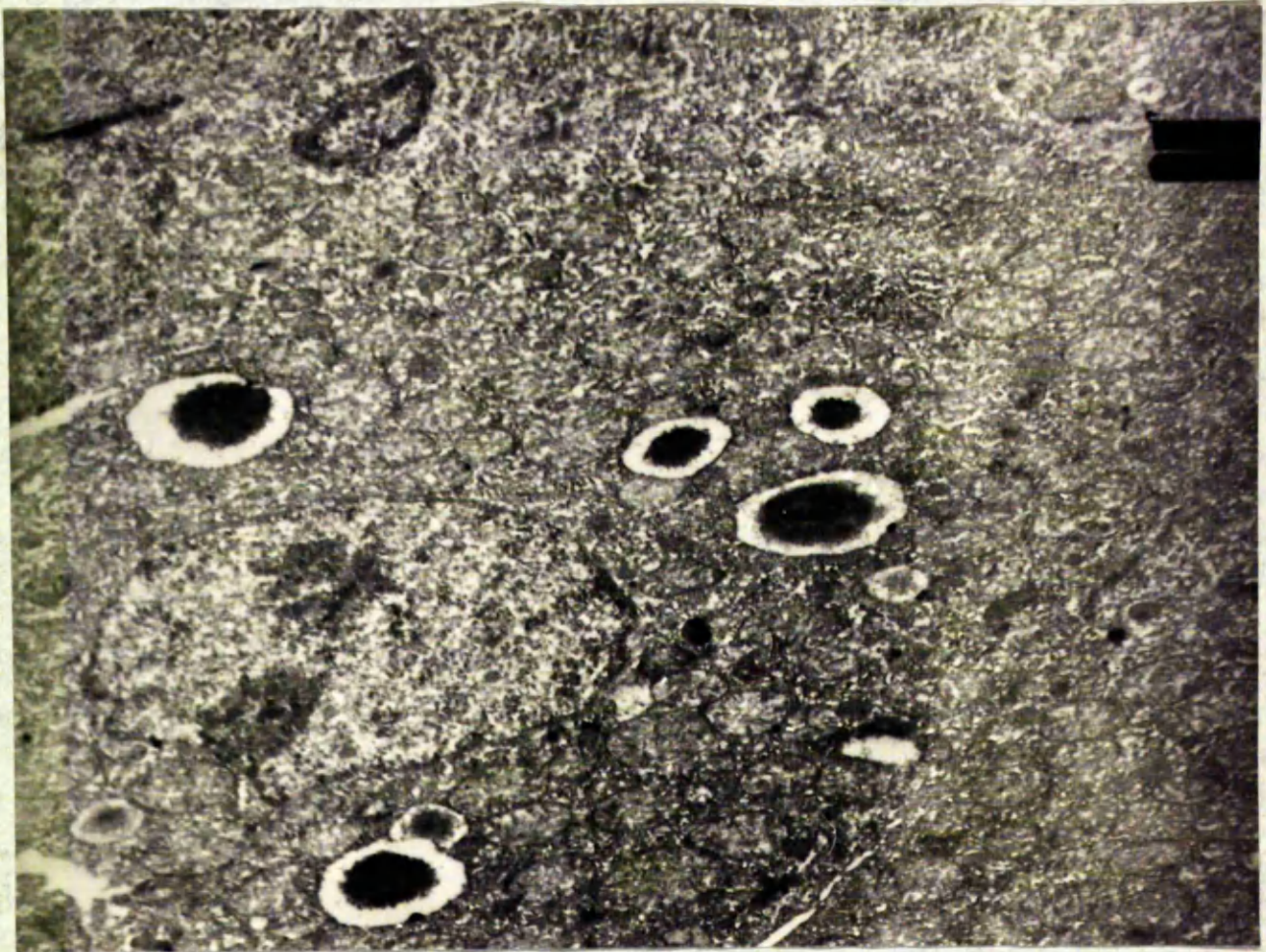
FIGURE 17



Magnification x 10,000

Electron micrograph of liver tissue from rat maintained on protein-deficient diet, and fasted overnight.

FIGURE 18



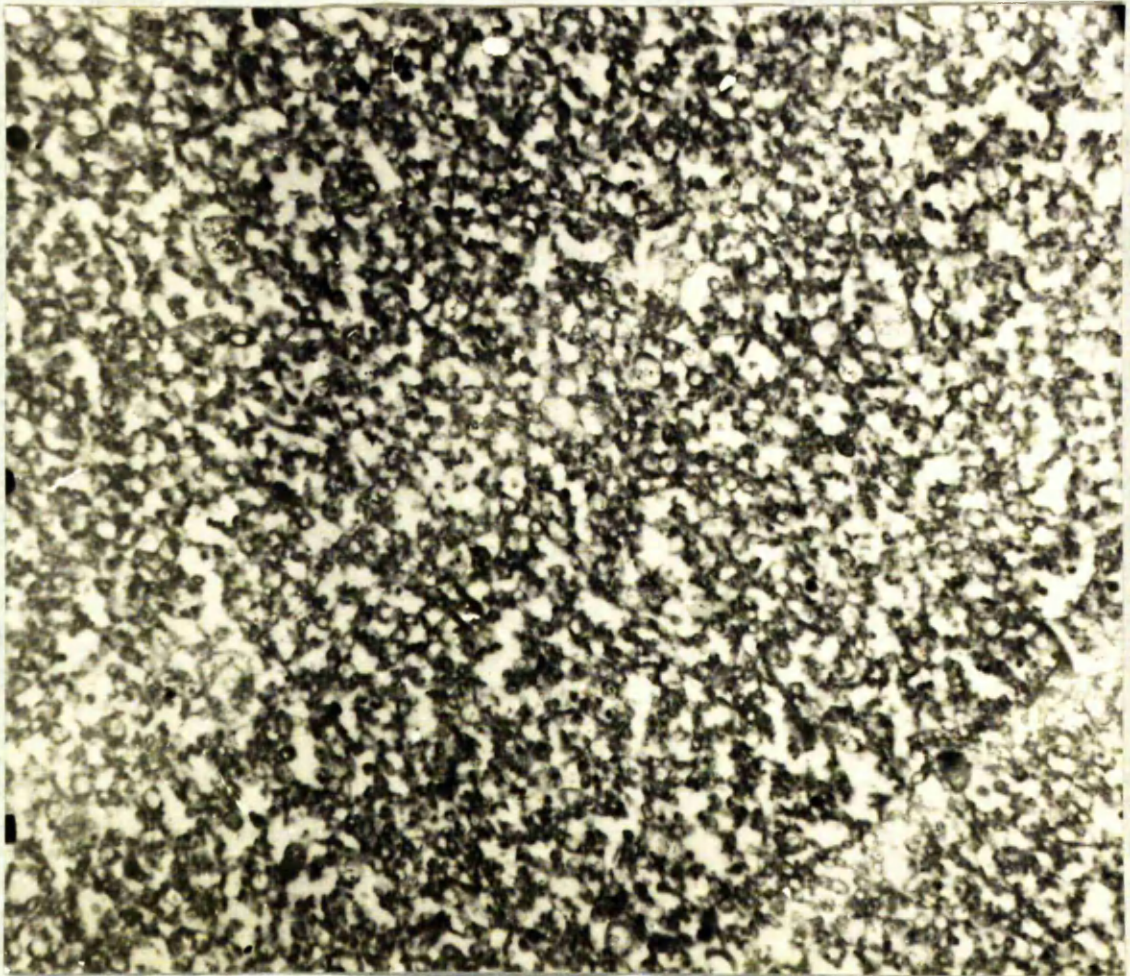
Magnification x 10,000

Electron micrograph of liver tissue from rat maintained on protein-deficient diet, fasted overnight and then given a protein meal 2 hours before being killed.

(Stenram, 1958). Both these animals had been fasted for 18 hours before death, so that partial breakdown of the endoplasmic reticulum might well have occurred during that period. Certainly the liver of a third rat, maintained on the same "low protein + carbohydrate" diet as the rat of Figure 17, but given a protein meal 2 hours before death, shows a more organised and granular reticulum (Figure 18). Figure 18 also shows that the nucleoli of protein-deficient rat liver are still prominent 2 hours after a protein meal. It is interesting to note that this micrograph shows mitochondria clustered near the nucleus. A similar concentration of mitochondria was observed by Bernhard and Kouiller (1956) in animals fed protein after a long period of starvation. In general, however, the more obvious of the differences seen in the micrographs are related to dietary treatment shortly before death, rather than to differences between the diets used for long-term maintenance.

The results of microscopic examination of whole tissue were confirmed by study of microsomal pellets prepared from the livers of rats maintained for 11 days on "HP + carbohydrate" or "LP + carbohydrate" diets, and fasted overnight before death. Figures 19 - 22 show that corresponding pellets from rats on different diets are not very different in appearance, but that those from the "HP + carbohydrate" group are a little denser. The micrographs also show that the difference between the "heavy" and "light" microsomes lies chiefly in the size of the vesicles. The vesicles of the "light" microsomes are smaller than those of the "heavy" microsomes, but they are quite prominent. The "light" microsomes thus do not consist merely

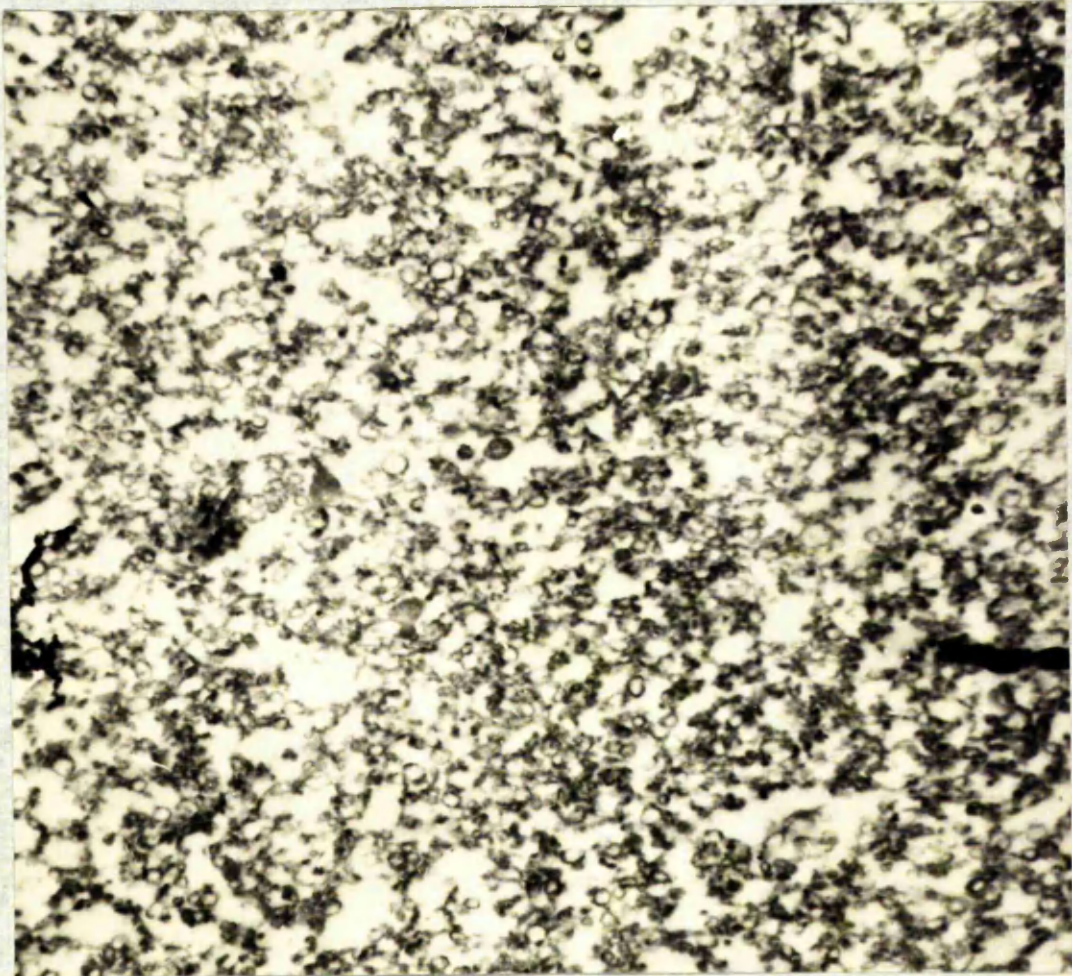
FIGURE 19



Magnification x 16,000

Electron micrograph of "heavy microsomal pellet" (8,500g fraction) prepared in 0.25M sucrose solution from liver of rat maintained on diet containing adequate protein, with glucose supplement.

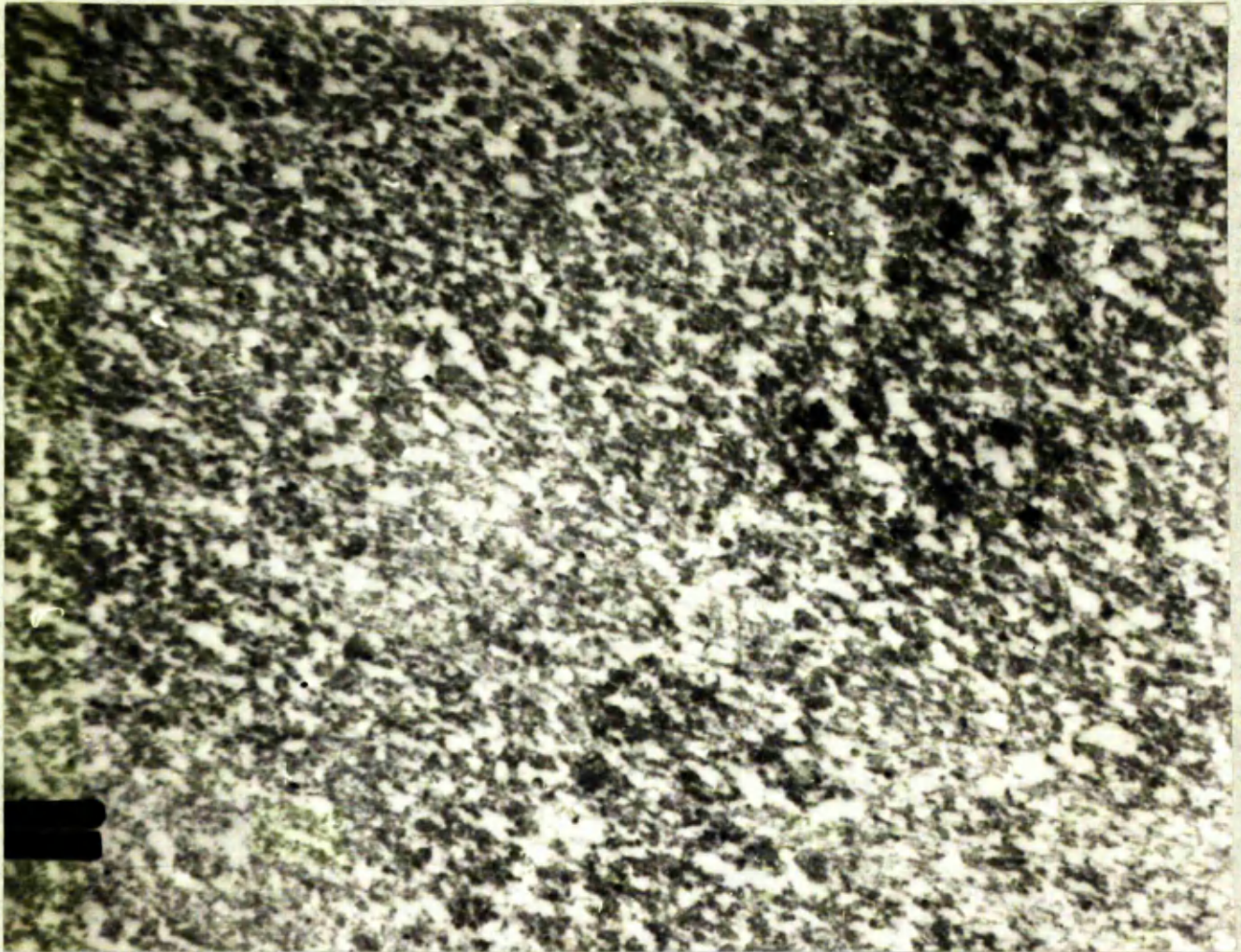
FIGURE 20



Magnification x 11,200

Electron micrograph of "heavy microsomal pellet" (8,500g fraction) prepared in 0.25M sucrose solution from liver of rat maintained on protein-deficient diet with glucose supplement.

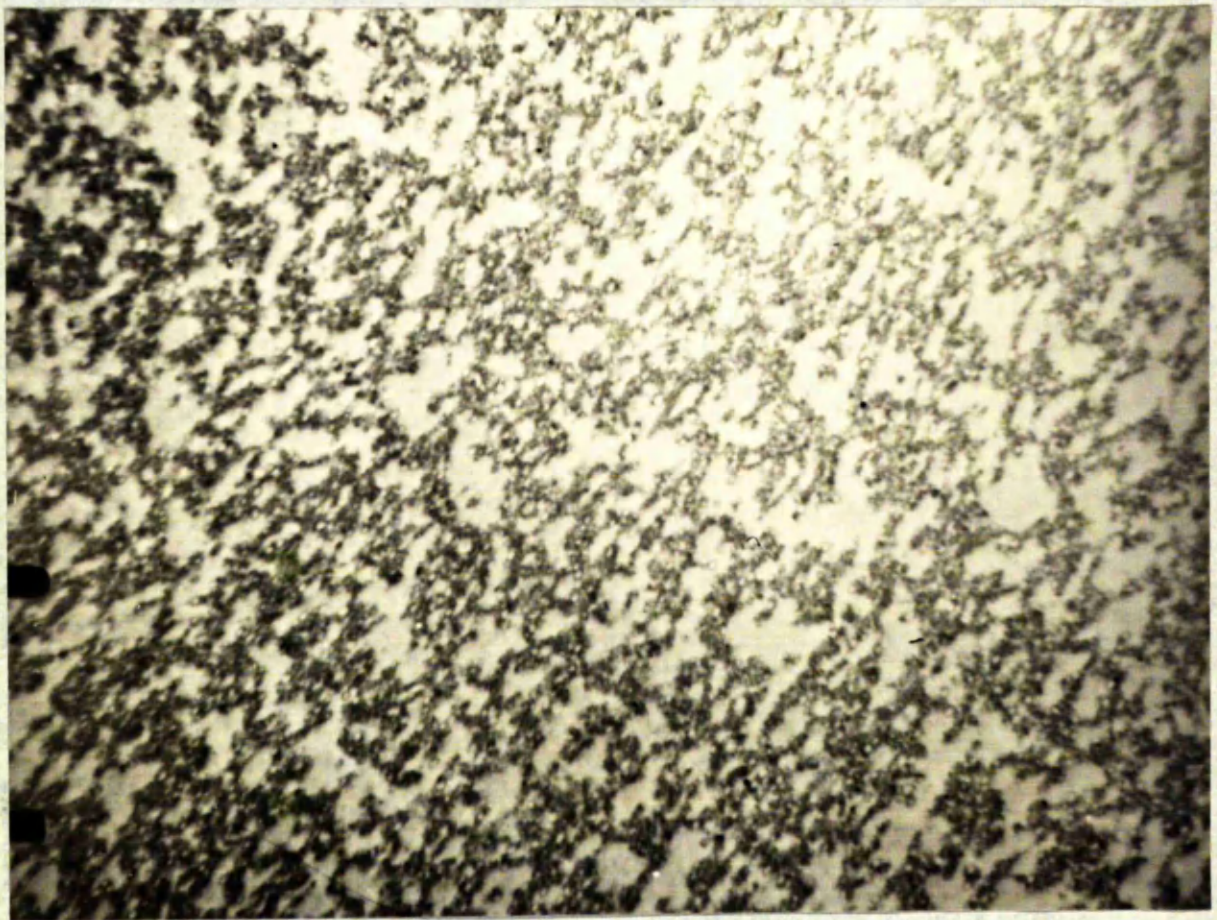
FIGURE 21



Magnification x 16,200

Electron micrograph of "light microsomal pellet" (18,000g fraction) prepared in 0.25M sucrose solution from liver of rat maintained on diet containing adequate protein, with glucose supplement.

FIGURE 22



Magnification x 10,700

Electron micrograph of "light microsomal pellet" (18,000g fraction) prepared in 0.25M sucrose solution from liver of rat maintained on protein-deficient diet with glucose supplement.

of ribosomal granules. This is as one would expect from the analytical data in Tables 15 and 19, which show that the "light microsomes" contain a certain amount of lipid protein and phospholipid as well as non-lipid protein and RNA.

We were thus unable to demonstrate any specific loss of "lipid N" from the liver microsomes of rats maintained for 11 days on protein-deficient diets. Our electron microscope studies, moreover, indicated that, while prolonged protein deficiency might lead to some reduction in the amount of endoplasmic reticulum in the liver cell, the decrease was not so gross as that observed by Fawcett (1955) and Bernhard and Rouiller (1956). The conditions which they used, however, were more drastic than ours, since their animals were fasted completely for 6 days. It therefore seemed possible that we might obtain a greater effect on the "lipid N" by carrying out experiments on rats fasted for several days. Furthermore, Fawcett noted that if the fasted rats were given a meal of protein, regrowth of the endoplasmic reticulum could be observed by electron microscopy within 6 hours of the protein meal; the new reticulum consisted of a membrane, initially without any attached granules. An increase in the "lipid N" of the microsome fraction might be expected to accompany such regrowth. We therefore carried out further investigations into a possible relationship between the "lipid N" and the endoplasmic reticulum by examining the effect of complete fasting and of protein re-feeding on the amount of "lipid N". The conditions used were as follows:

- (a) Rats were fasted overnight for 18 hours, and killed either fasting or 1½ hours after a protein meal. After 18 hours' fasting the endoplasmic reticulum should be breaking down, and the protein meal should reverse the process (Munro and Clark, 1960).
- (b) Rats were fasted completely for 5 days, and killed either fasting or shortly after receiving a protein meal. Of those given the protein meal, some were killed 1½ hours later and others 6 hours later. The latter time corresponds to that at which Fawcett first observed regrowth of reticulum in rats fed protein after prolonged fasting.

In the case of the animals (a) fasted overnight, Table 20 shows that the protein-fed and the fasted series contain the same total amount of "lipid N" in the combined microsomal fractions ("light" and "heavy") and the cell sap, taken together. The fasted series, however, contains slightly less in the microsomes and more in the cell sap. This is to be expected if during fasting the reticulum breaks down into small fragments, and this disintegration is reversed by a protein meal, since the fragments are probably associated with the cell sap.

The rats (b) fasted for 5 days showed rather larger increases in "lipid protein" after a protein meal. In this case the fraction analysed consisted of "heavy microsomes" only, and an increase in the amount of "lipid protein" can be seen both 1½ hours and 6 hours after a protein meal. The lipid-free protein fraction shows no increase.

The electron micrographs of the livers of these rats (Figures 23 and 24)

TABLE 20

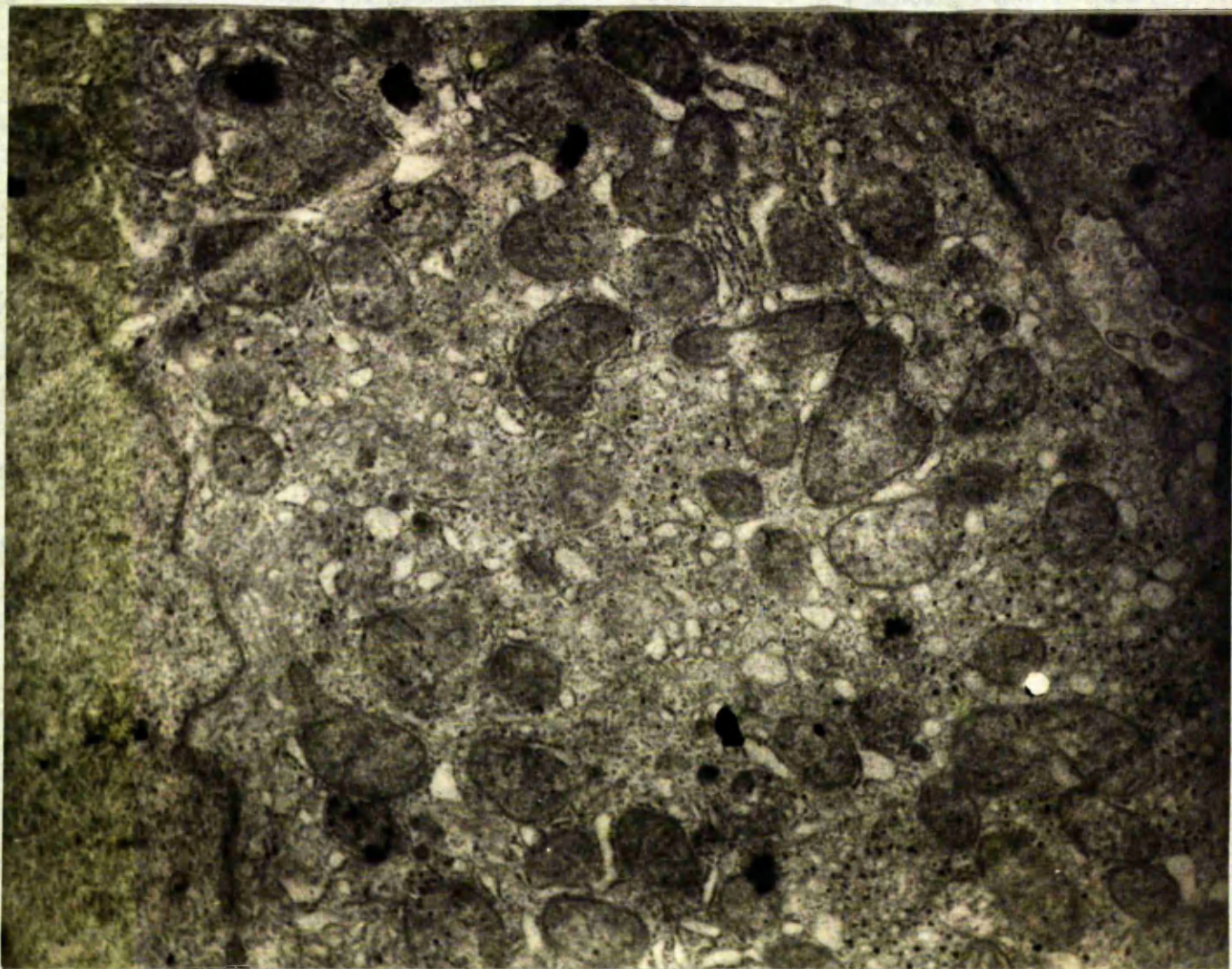
Effect of dietary state on protein content
of microsomes and cell sap

The results are expressed as mg. protein in the microsomal pellet derived from 100 mg. liver.

Results	Time fasted	Time after meal	No. of animals	Microsomes		Cell sap	
				Fed	Not fed	Fed	Not fed
Lipid protein	18 hours	1½ hours	3 [±]	0.45	0.39	0.60	0.63
	5 days	1½ hours	2	0.75	0.53	--	--
	5 days	6 hours	1	0.65	0.50	--	--
Non-lip. protein	18 hours	1½ hours	3 [±]	1.31	1.34	--	--
	5 days	1½ hours	2	1.31	1.59	--	--
	5 days	6 hours	1	1.89	1.92	--	--

[±] The microsomal preparation in this series included "light microsomes" and "heavy microsomes". The pellets and cell sap were treated with deoxycholate (DOC), and the results are calculated as the sum of the DOC-soluble and DOC-insoluble fractions. In the other series (fasted for 5 days), the "microsomes" analysed were untreated "heavy microsomes".

FIGURE 23

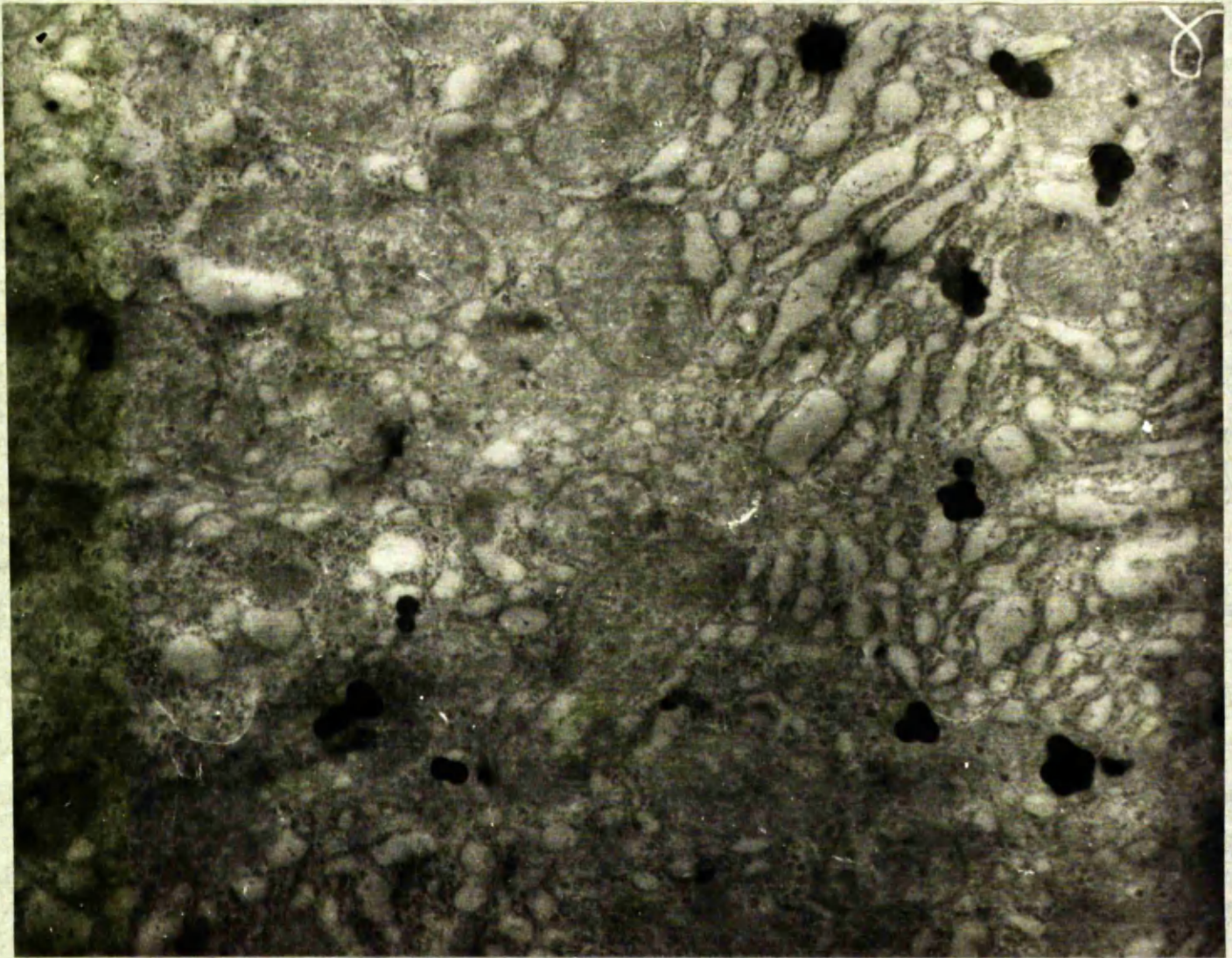


Magnification x 11,700

Electron micrograph of liver tissue from rat fasted for 5 days.

Stained with uranyl acetate.

FIGURE 24



Magnification x 21,100

Electron micrograph of liver tissue from rat fasted for 5 days,
and given a protein meal 6 hours before being killed.
Stained with uranyl acetate.

show that the endoplasmic reticulum of the liver cells in the animals fasted for 5 days does seem to be diminished, although not completely absent. Six hours after a protein meal there appears to be a regrowth of the reticulum (Figure 24). This finding agrees with the observation of Fawcett that the regeneration of the reticulum in fasting rats could first be seen about 6 hours after a protein meal.

Our findings may be summarised as follows:

1. The protein-like material removed from TCA-treated rat liver is a major component of the liver cell of both the rat and the mouse.
2. The greater part of the "lipid protein" is contained in the particulate fractions of the cell; the percentage of the total protein of each fraction which is "lipid protein" is highest in the mitochondria and heavy microsomes (the membranous fractions).
3. In rats fasted overnight, the cell sap contains more "lipid protein" than the cell sap of unfasted mice. This high content in the cell sap in the fasted animals may be due to fragments of disintegrated endoplasmic reticulum, which breaks down during fasting.
4. A diet deficient in protein or in calories causes a reduction in the amount of all the constituents (except DNA) estimated in the whole cell, but the percentage composition of the cell is not greatly affected by these dietary variations.
5. These deficient diets also reduce the total weight of each of the cytoplasmic fractions, without altering their composition. Thus the cell fractions are diminished as a whole, without specific loss of any constituent.

6. Electron microscope studies showed that liver from rats maintained on a protein-deficient diet contained a little less endoplasmic reticulum than the liver from rats maintained on a diet containing adequate protein. The protein-deficient liver showed the large nucleolus characteristic of protein deficiency. In rats maintained on the protein-deficient diet but given a protein meal 2 hours before death, the endoplasmic reticulum had a more granular and organised appearance. Even in the protein-deficient rat, the reticulum was not completely absent, and examination of cell fractions prepared in sucrose confirmed that the microsomal pellets ("heavy" and "light") from the protein-deficient rats were similar to those from the adequately-fed rats, except that they were rather less dense.
7. Examination in the electron microscope of "heavy" and "light" microsomal pellets confirmed the indications from analysis of these fractions that the "light microsomes" are not identical with "ribosomes". They are rich in granules, but contain vesicles as well; the vesicles are smaller than those of the "heavy microsomes", and the chief difference between the two microsomal fractions seems to lie in the number and size of the vesicles.
8. A rat fasted for 18 hours and then given a protein meal 1½ hours before death has more "lipid protein" in the liver "heavy microsomes" and less in the cell sap than a similarly fasted rat not given protein. This is in agreement with the findings of Munro and

Clark, that the endoplasmic reticulum breaks down during fasting, and that the breakdown is reversed by a protein meal.

9. In rats starved for 5 days, larger increases in the microsomal lipid protein $1\frac{1}{2}$ and 6 hours after a protein meal have been observed. Electron micrographs show a reduction in the reticulum during protein depletion, and regrowth within 6 hours of a protein meal.

SECTION V

The subfractionation of cell fractions by chemical means

In Section III it was shown that the membranous fractions of the liver cell are particularly rich in "lipid N", and in Section IV evidence was presented which indicated a possible connection with the endoplasmic reticulum. Our next step was to treat the microsomes and some other fractions of the cell with some of the chemical reagents which have been used to separate the membranous and granular components of the microsomes, and to analyse the "lipid N" and non-lipid protein content of the resulting fractions.

The reagents used were

- (a) sodium pyrophosphate
- (b) sodium deoxycholate
- (c) lysolecithin
- (d) the α -toxin (haemolysin) from Staphylococcus aureus

(a) Sodium pyrophosphate:

This reagent removes the ribosomal granules from the surface of the endoplasmic reticulum, and reduces them to fragments no longer sedimentable on centrifugation (Sachs, 1958). If our lipid protein is a component of the membranes only, it should be spun down with the membranous pellet after pyrophosphate treatment.

Preparations of heavy microsomes were treated at 0° with 0.10M pyrophosphate in sucrose solution at pH 7.4 for various lengths of time. The sample which was suspended in ice-cold sucrose alone for two hours was

taken as a control, and Table 21 and Figure 25 illustrate the loss of various constituents on treatment with pyrophosphate. The length of time of treatment does not seem to be important, as the effects of exposure for two hours before centrifugation are no greater than those of exposure during the period of centrifugation only. The chief effects are on the RNA and on the non-lipid protein, which fall to 44% and 69% respectively of the control values. The other components do not seem to be much affected by pyrophosphate; phospholipid is not reduced by short periods of treatment, although it may fall a little if the time of exposure is prolonged, and lipid protein does not seem to be affected even by 2 hours' treatment with pyrophosphate. The composition of the fraction removed by pyrophosphate can be calculated by difference, and proves to be 31% RNA and 69% non-lipid protein. It thus appears to consist largely of ribosomes (which contain 40% RNA and 60% protein), slightly contaminated by non-lipid protein. These results indicate that the response of the lipid protein to pyrophosphate treatment follows that of the phospholipid of the membrane rather than that of the non-membranous components, such as the non-lipid protein.

One might therefore expect that the increase in microsomal "lipid protein" produced in the fasting rat by a protein meal (see Table 20) would be confined to the pyrophosphate-insoluble (membranous) fraction. The results in Table 22 indicate that this is so, since the increase in the "lipid N" content of the control (sucrose-washed) samples produced by feeding a protein meal ($0.53 - 0.45 = 0.08$ mg.) is wholly accounted for by the

TABLE 21

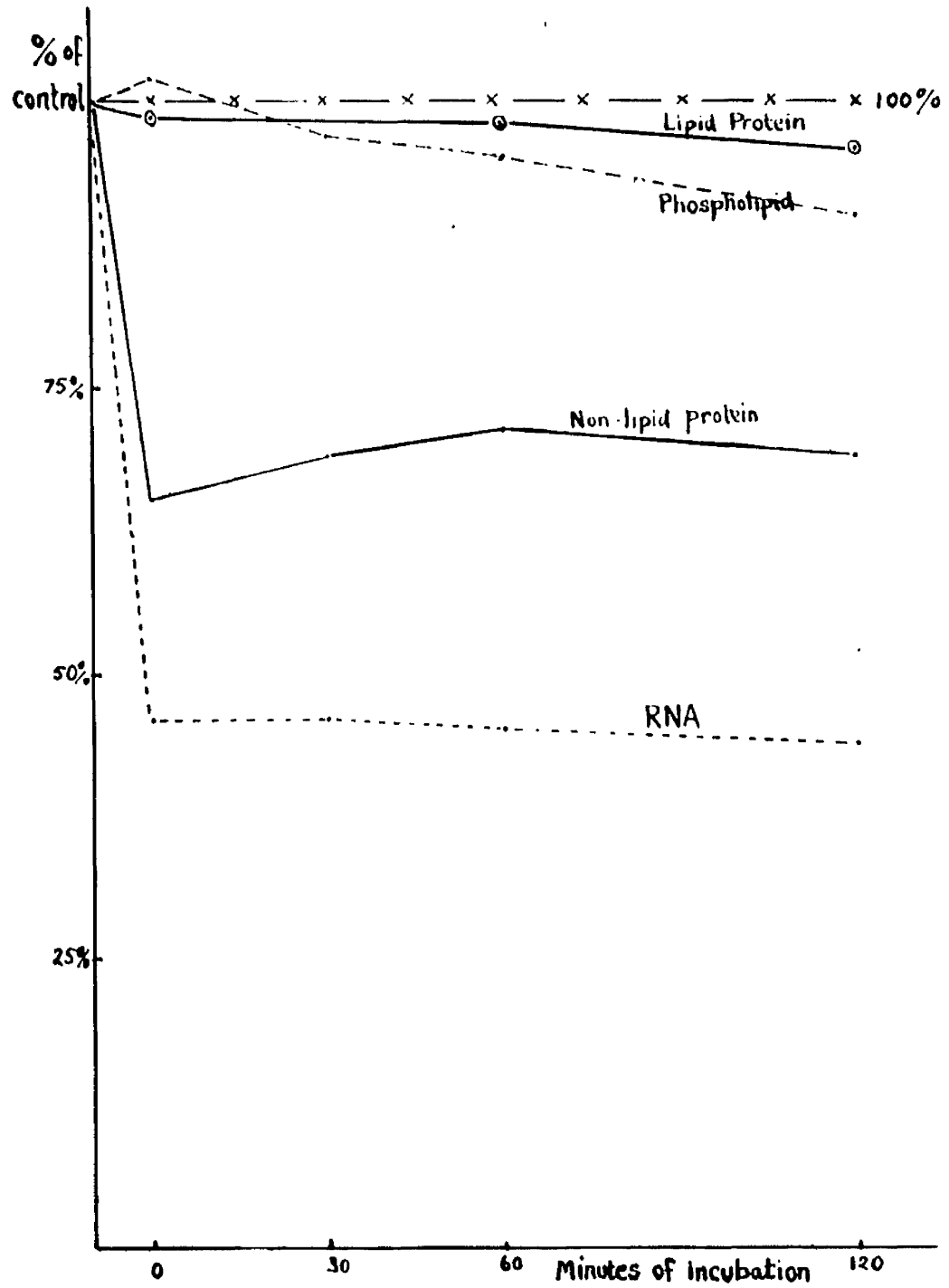
Composition of pellet obtained from microsomes by treatment for various times with pyrophosphate (PP)

The results, which are the average data from two experiments, are expressed as mg. in the pellet derived from 100 mg. liver.

Treatment	Time of incubation (min.) ¹	RNA	Phospholipid	Lipid protein	Non-lipid protein
sucrose	0	0.26	0.74	0.61	1.48
sucrose	120	0.255	0.74	0.56	1.58
sucrose/PP	0	0.12	0.76	0.56	1.03
sucrose/PP	30	0.11	0.71	0.49	1.07
sucrose/PP	60	0.11	0.70	0.55	1.10
Sucrose/PP	120	0.11	0.67	0.54	1.09

¹ * further 60 minutes' exposure during centrifugation

FIGURE 25



Effect of pyrophosphate treatment on microsomes

TABLE 22

The effect of fasting and protein-feeding
on the pyrophosphate-insoluble fraction of microsomes

Four rats were fasted for 5 days, and two were then given a protein meal. All the rats were killed two hours later, and "heavy microsome" fractions were prepared from the pooled homogenate of each pair of livers. Samples were treated for 30 minutes at 0° with pyrophosphate in sucrose solution or with sucrose solution alone, and then spun for 60 minutes at 105,000g. The pellets were analysed as usual. The results are expressed as mg. in the pellet from 100 mg. liver.

Treatment	Sucrose washed (control)		Pyrophosphate- treated	
	meal + 2 hours	no meal	meal + 2 hours	no meal
mg. per 100 mg. liver:-				
Lipid protein	0.53	0.45	0.47	0.38
Non-lipid prot.	1.19	1.19	0.89	0.84
Phospholipid	0.55	0.47	0.54	0.48
RNA	0.22	0.22	0.11	0.13
Ratios:-				
Lipid protein/ non-lipid prot.	0.45	0.38	0.53	0.45
Lipid protein/ phospholipid	0.98	0.98	0.87	0.84
non-lipid prot/ phospholipid	2.17	2.52	1.65	1.75

increase in the pyrophosphate-insoluble fraction ($0.47 - 0.38 = 0.09$ mg.). In agreement with Table 21, pyrophosphate treatment causes only a slight fall in the "lipid N" content, and none in the phospholipid content. The phospholipid content shows a small increase after a protein meal; its response to protein feeding is thus similar to that of the "lipid N", as is indicated by the lack of change in the lipid protein/phospholipid ratio. Table 22 also shows clearly the divergence between those constituents which are insoluble in pyrophosphate, and the pyrophosphate-soluble fraction of the microsomes. Thus, as was found in Table 21, both RNA and non-lipid protein are partly removed by pyrophosphate, and neither quantity is increased by a protein meal. The differences in the behaviour of "lipid" and "non-lipid" protein may be seen in a comparison of the ratios of lipid protein/phospholipid and non-lipid protein/phospholipid. The behaviour of the lipid protein resembles that of the phospholipid rather than that of the non-lipid protein.

(b) Sodium deoxycholate:

The effect of deoxycholate (DOC) is the converse of that of pyrophosphate, as it dissolves the membranous component of the microsomes, leaving the granules. The form of treatment used was that of Littlefield, Keller, Gross and Zamecnik (1955), and the microsomal fraction to be treated was prepared by their method, so that we could make comparisons with their published data. The fraction was roughly equivalent to the combined "heavy" and "light" microsomes of our own scheme. After DOC treatment, the pellets and soluble fractions were precipitated and washed with TCA,

TABLE 23

Effect of deoxycholate on the protein of rat liver microsomes

Microsomes were prepared and treated with DOC as described by Littlefield, Keller, Gross and Zamecnik (1955), and the fractions analysed as usual. The results are the mean data from the analysis of three livers, and are expressed as mg. in the microsomes from 100 mg. liver. The figures in brackets represent the percentage of the total protein in each fraction which is "lipid" or "non-lipid".

Fraction	Lipid protein	Non-lipid protein	Total protein
DOC-soluble	0.34(27%)	0.93(73%)	1.27
DOC-insoluble	0.07(15%)	0.39(85%)	0.46

and extracted with the usual lipid solvents. The distribution of "lipid N" and non-lipid protein in the different fractions is shown in Table 23. The "lipid N" seems to be preferentially soluble in DCC, which dissolves 83% of the total "lipid protein" but only 70% of the non-lipid protein. These results again suggest that the "lipid N" is associated with the membranous part of the microsomes. Analysis of the DCC-insoluble pellet showed that it contained 45% RNA, and was probably therefore a fairly pure preparation of ribosomes. Such ribosomes are essentially devoid of "lipid N".

(c) Lysolecithin:

This haemolytic reagent has been shown (Robinson and Saunders, 1959) to have a solubilising action on lipids (triolein, etc.), and may therefore be expected to attack the membranous part of the microsomes. Our initial experiments with lysolecithin were carried out on whole microsomes. Owing perhaps to the technical difficulty of dispersing the lysolecithin (which was initially dissolved in chloroform/methanol) in the aqueous suspension of microsomes, the results were inconsistent. In experiments where lysolecithin did appear to have some effect in dissolving the lipid protein, it generally seemed to affect the non-lipid protein equally; it was not possible to measure its effect on phospholipid since the phosphorus content of lysolecithin itself interfered in the estimation. It was, therefore, thought that evidence of more specific action of lysolecithin might be obtained by using membrane preparations made with pyrophosphate. Microsomes were treated sequentially with pyrophosphate and lysolecithin according to the scheme outlined in Figure 26. Figures 27-9 show the appearance

FIGURE 26

Treatment of rat liver microsomes with pyrophosphate
and lysolecithin to provide pellets referred to in Table 24

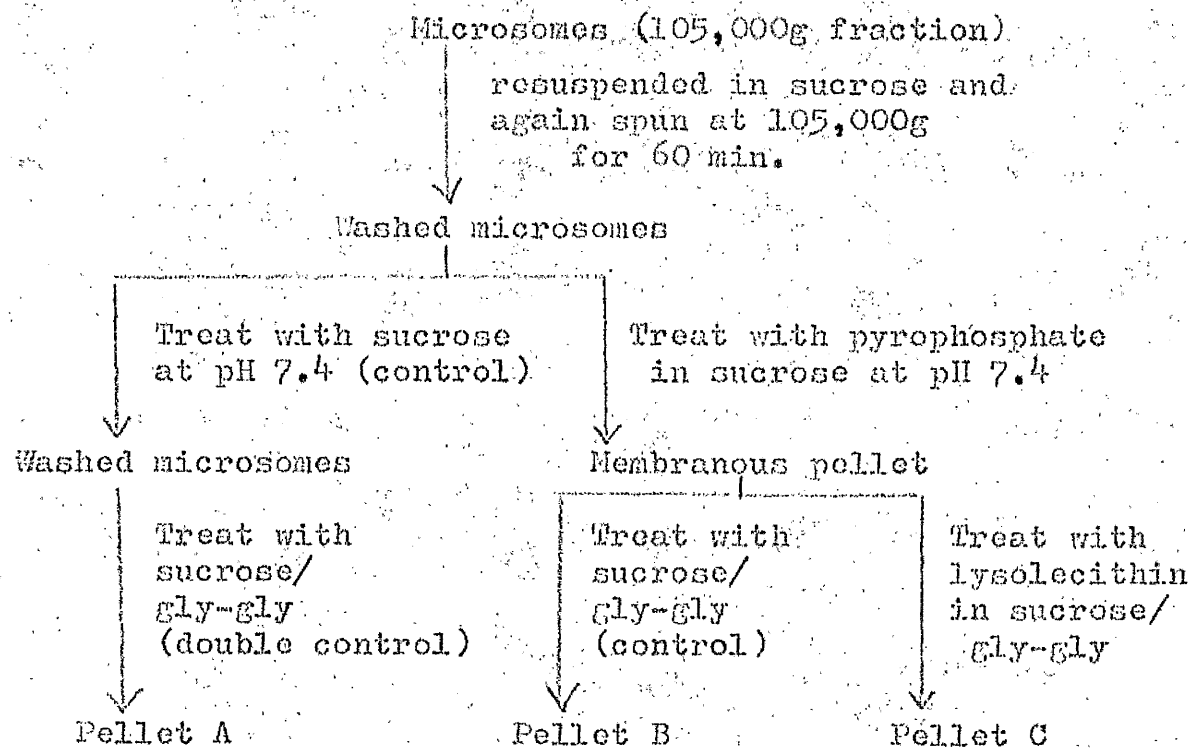
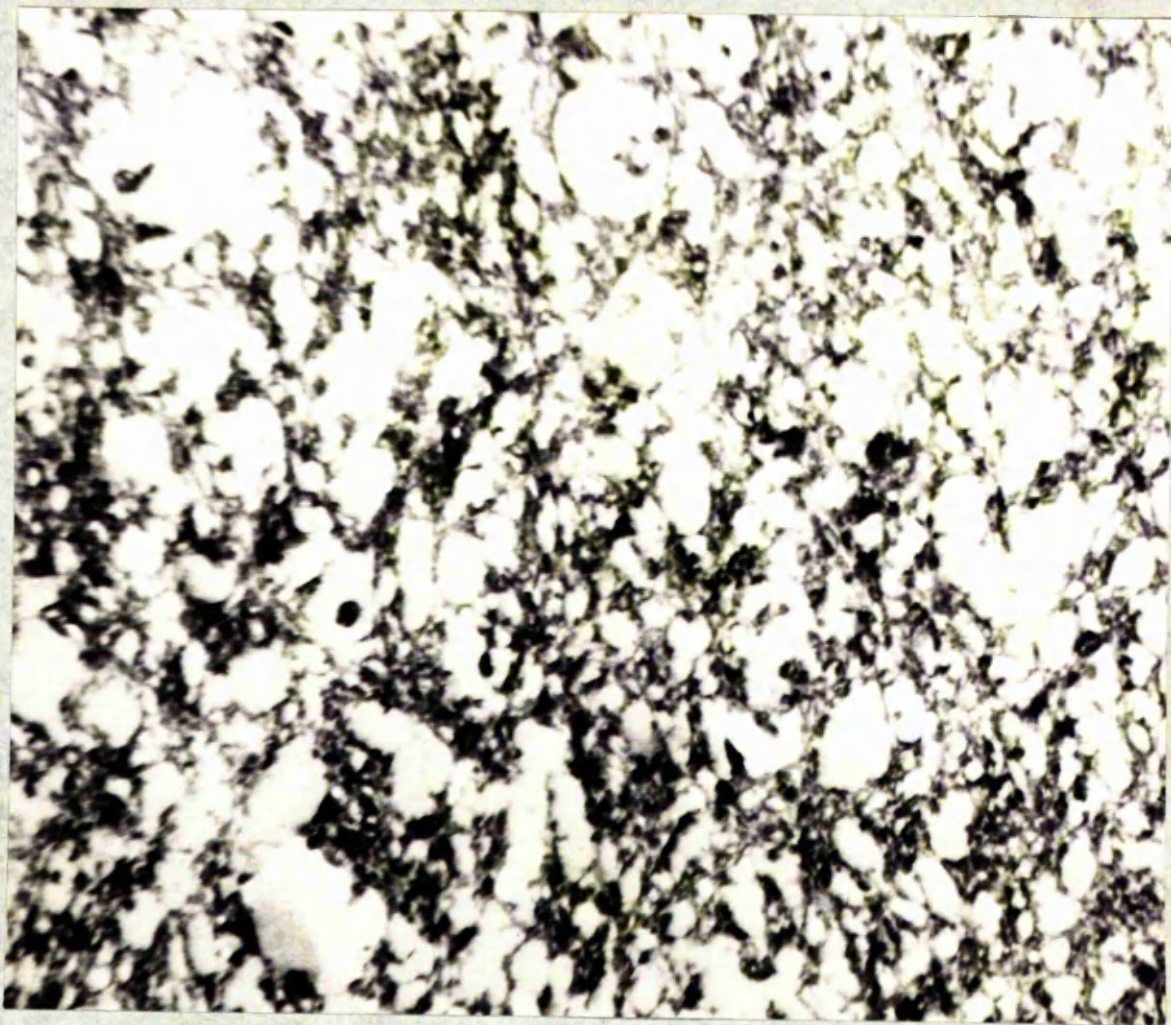


FIGURE 27

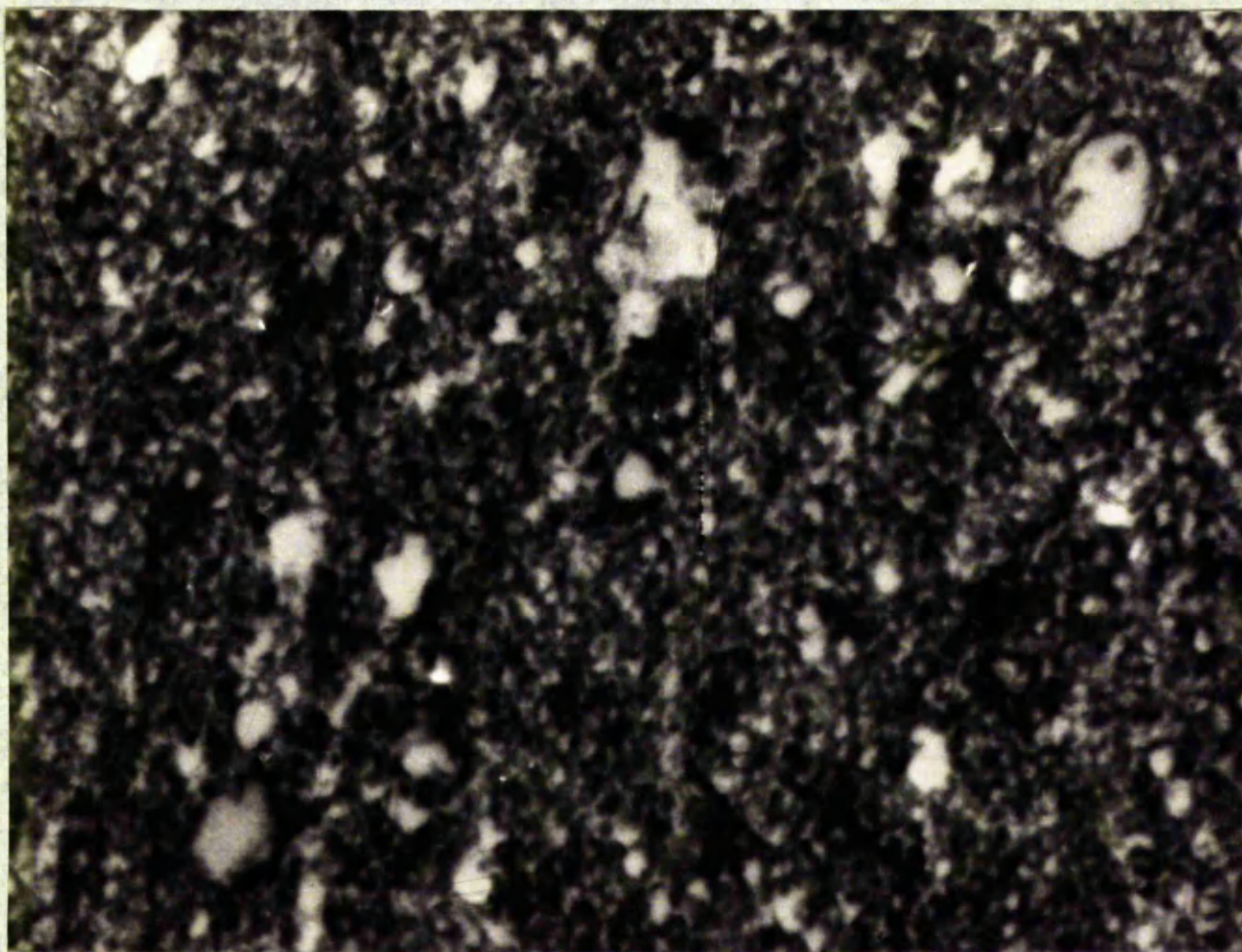


Magnification x 23,000

Electron micrograph of microsome pellet treated with sucrose
and then with sucrose/glycyl-glycine as in Figure 26.

(Pellet A)

FIGURE 28

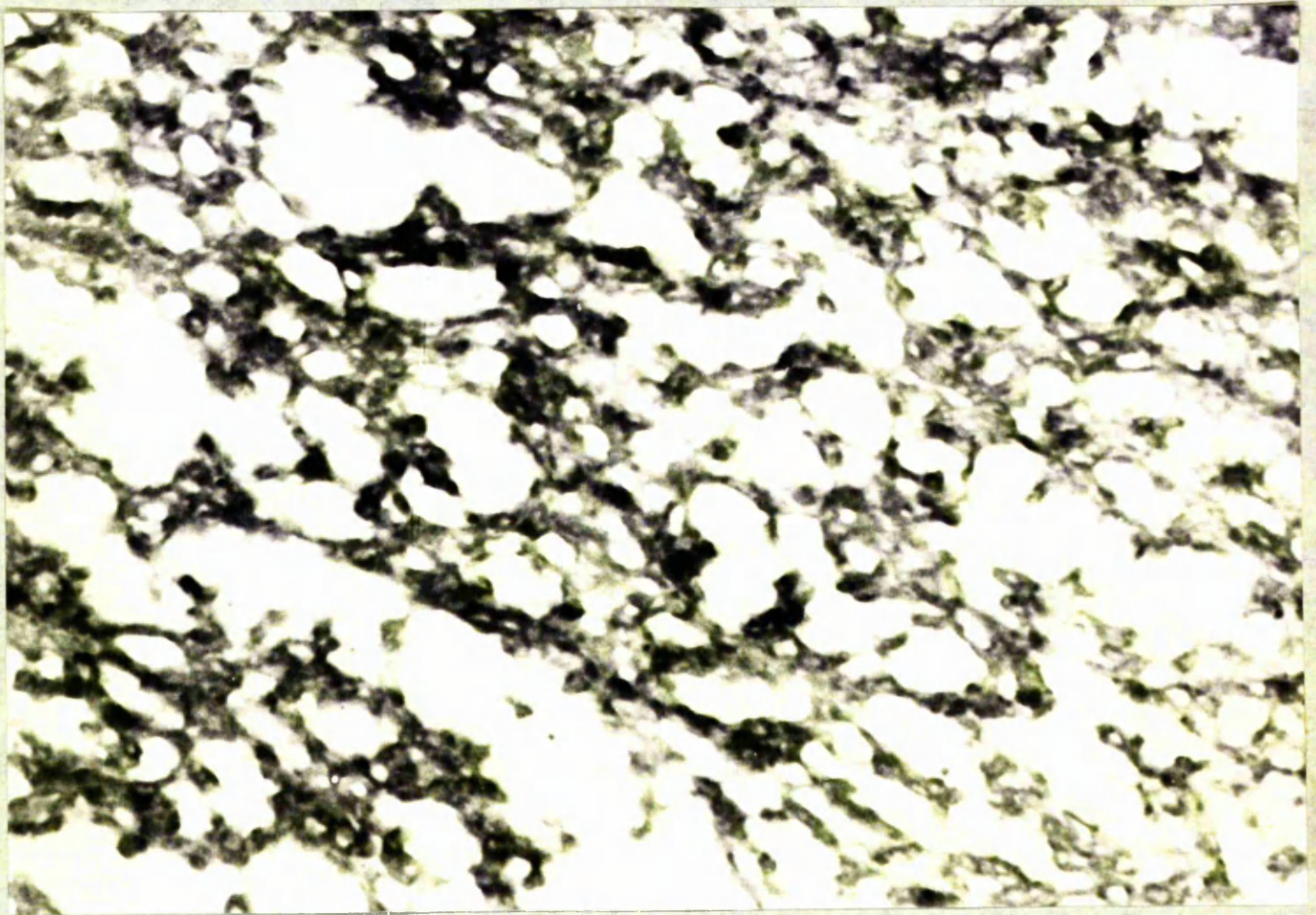


Magnification x 37,000

Electron micrograph of microsome pellet treated with pyrophosphate and then with sucrose/glycyl-glycine, as in Figure 26.

(Pellet B)

FIGURE 29



Magnification x 36,000

Electron micrograph of microsome pellet treated with pyrophosphate and then with lysolecithin, as in Figure 26.

(Pellet C)

of the resulting pellets in the electron microscope. These micrographs are not so revealing as might have been hoped, but some difference between the different pellets can be seen. The control pellet A (washed with sucrose and sucrose/glycyl-glycine only) contains vesicles studded with dense granules. In pellet B, pyrophosphate has removed most of the granules, leaving a dense and fairly uniform mass of membranous material, which appears to have re-formed into smaller vesicles. The few larger vesicles which remain still show attached granules, and may have escaped the action of pyrophosphate. In the pellet C, which was treated with pyrophosphate and lysolecithin, the granules are, of course, absent (owing to the treatment with pyrophosphate), but the subsequent treatment with lysolecithin has further altered the appearance of the membranous material also. It has a very open structure with large holes, and appears in many places as a coagulated mass.

The analytical data for these three types of pellet are given in Table 24. They indicate that, as would be expected in view of the results in Table 21, the contents of both RNA and non-lipid protein have been considerably reduced by treatment with pyrophosphate (Pellet B). In this experiment, subsequent treatment with lysolecithin removed about 29% of the lipid protein of the pyrophosphate-insoluble fraction of the microsomes without greatly affecting its remaining non-lipid protein. All these findings are in good agreement with the electron microscope picture, and support the theory that the lipid protein is a component of the microsomal membrane. On the whole, however, the effect of lysolecithin proved to be unpredictable.

TABLE 24

Effect of lysolecithin on
pyrophosphate-treated rat liver microsomes

Microsomes prepared and treated with pyrophosphate (PP) as described by Littlefield *et al* (1955) were then treated with either lysolecithin (LL) in a sucrose/gly-gly medium or with the medium alone (see Figure 26). Another series of microsomal pellets were treated as a double control, i.e. they were treated with sucrose medium instead of with pyrophosphate, and then with sucrose/glycyl-glycine medium instead of with lysolecithin. The results of this experiment are expressed as mg. in the pellet derived from 100 mg. liver, and also as a percentage of the values found in the double control series (A). They are the mean data from analysis of three homogenates of liver from a single rat (or two homogenates in the case of Pellet B).

Treatment	Protein		Phospholipid		RNA	
	lipid wt. % of A	non-lipid wt. % of A	wt. % of A	wt. % of A	wt. % of A	wt. % of A
Control (A)	0.32 (100%)	1.54 (100%)	0.73 (100%)	0.295 (100%)		
PP + medium (B)	0.45 140%	1.06 69%	1.02 140%	0.062 21%		
PP + LL (C)	0.23 71%	0.91 59%	- -	0.042 14%		

Note: The high results obtained in Pellet B for the content of lipid protein and phospholipid (140% of the control values) suggest that all the figures for Pellet B may be 40% too high. (Pellet B estimations are based on two samples only). If the RNA and non-lipid protein figures are multiplied by the factor 100/140, they approach quite closely to the values obtained for Pellet C.

and this reagent did not provide a satisfactory means of fractionating microsomes.

(d) Haemolysin (α -toxin) from *Staph. aureus**:

This toxin lyses red cell membranes, which are lipoprotein structures, and it was thought that it might also dissolve microsomal membranes, and thereby provide a useful means of fractionating microsomes.

At first the microsomes were prepared in the usual way in sucrose solution, and sucrose was also present in the medium during incubation with the toxin. When the toxin in these conditions appeared to have no effect on any of the four constituents measured, an inhibitory effect of sucrose was suspected (since glucose was known to inhibit the haemolytic activity of the toxin, and sucrose was subsequently shown to do so also). In later experiments, therefore, the microsomes, after preparation in sucrose as usual, were washed and incubated in Krebs-Ringer bicarbonate buffer solution. The results obtained by the use of these conditions are shown in Table 25. It is clear that, while the toxin removes lipid protein, it also removes a similar proportion of the other constituents, with the possible exception of phospholipid. Thus, while the toxin does affect the microsomes, it probably has a non-selective action.

Since these effects were observed after an hour's incubation, however, it seemed likely that they were the result of total disintegration of the microsomal structure, and that by the use of shorter incubation times it

* These experiments were carried out in collaboration with Dr. I. R. W. Lominski and Mr. J. Arbuthnott, who provided the preparations of active and inactivated toxin.

TABLE 25

The microsomes were washed with bicarbonate buffer and incubated in sucrose-free medium with active or inactivated (heated) toxin. "Blank" tubes containing toxin but no microsomes were also incubated and analysed, and the figures obtained for these tubes have been subtracted from those of the appropriate "Test".

The results are derived from the analysis of duplicate samples, and are expressed as mg. in the pellet derived from 100 mg. of liver. The figures in brackets represent the content of the toxin-treated pellet as a percentage of the amount in the pellet treated with inactive toxin.

Treatment	Protein		RNA	Phospholipid
	lipid	non-lipid		
Treated with heat-inactivated toxin	0.53	1.26	0.26	0.78
Treated with active toxin	0.39(74%)	0.995(79%)	0.20(76%)	0.70(90%)

might be possible to stop the process at a stage where certain components had been dissolved more than others. Such a finding would imply a sequential breakdown of the microsomes. Table 26 shows the results of incubating microsomes with toxin (in bicarbonate buffer) for various times over the range 10 minutes to 2 hours. There is little evidence of sequential breakdown, since, with the possible exception of phospholipid, the various constituents seem to be dissolved at a constant rate over 2 hours, as can be seen from Figure 30.

Comparison of the effect of active toxin with that of toxin inactivated by autoclaving, however, indicates that part of the solubilisation process is probably due to the conditions of incubation and not to any specific action of the active toxin. Thus, although the lipid protein is reduced to 69% of its initial value (from 0.45 to 0.31) after 2 hours' incubation with active toxin, the same is true of the series in which heated toxin was used (0.46 to 0.32). When the content of the pellets after 2 hours' incubation with active toxin is calculated as a percentage of the amount in the samples treated for the same period with inactivated toxin, it becomes clear that only the non-lipid protein and the RNA are removed specifically by the action of the toxin. The lipid protein and phospholipid are much less affected.

These rather unexpected results suggest that any specific effect the Staph. aureus toxin may have on microsomes is on the ribosomal granules and not, as might have been predicted from its effect on the stromata of red cells, on the membranes. This implies that the toxin causes detachment

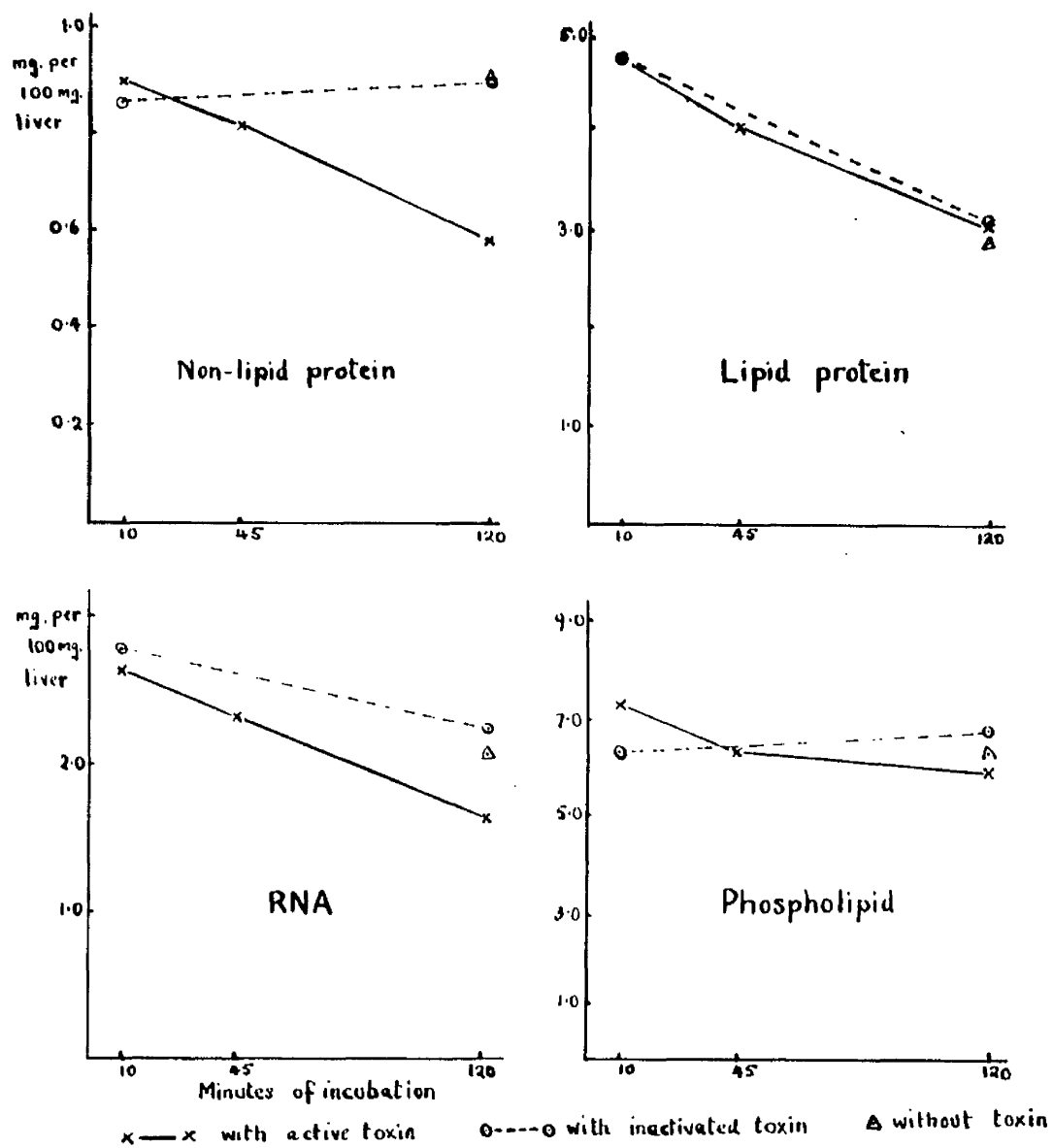
TABLE 26

Effect on microsomal pellet of treatment for various timeswith Staph. aureus haemolysin

Microsomes prepared in sucrose and washed with bicarbonate buffer were incubated in sucrose-free medium with active or inactivated toxin, or with the medium only, for the times stated. The results are based on analysis of duplicate portions of a single homogenate of liver from one rat, and have been corrected for the content of the "toxin blanks". They are expressed as mg. in the pellet derived from 100 mg. liver. The figures in brackets represent the content of the pellets treated with active toxin for 120 minutes as a percentage of the content of pellets treated for the same time with heat-inactivated toxin.

Time (min.)	Sample	Protein (lipid)	Protein non-lipid	phospho-lipid	RNA
10	Microsomes + inactivated toxin	0.46	0.87	0.64	0.21
	Microsomes + active toxin	0.45	0.91	0.73	0.26
45	Microsomes + active toxin	0.42	0.81	0.64	0.23
120	Microsomes only	0.28	0.91	0.64	0.21
	Microsomes + inactivated toxin	0.32(100%)	0.91(100%)	0.68(100%)	0.23(100%)
	Microsomes + active toxin	0.31(97%)	0.58(64%)	0.60(88%)	0.17(73%)

FIGURE 30



Effect of haemolysin on microsomes

of ribosomes from the membrane, possibly with partial disruption of the ribosomes.

As far as the source of the lipid protein is concerned, however, the results obtained by the use of this toxin show that on the whole the lipid protein follows the solubility pattern of the phospholipid, and seems likely to be part of the microsomal membrane.

Summary:

All four of the reagents we have used for the fractionation of microsomes (pyrophosphate, deoxycholate, lysolecithin and haemolysin) have had an effect on the lipid protein quite different from their effects on non-lipid protein. The reagents which attack the membrane (DOC and lysolecithin) specifically remove the lipid protein but not the non-lipid protein, while those which dissolve the ribosomal granules (pyrophosphate and possibly haemolysin) have the reverse action. In these fractionations the lipid protein solubility follows that of the phospholipid, and it seems probable that the lipid protein is a component of the membranous part of the microsomes. This would account for the fact that the content of lipid protein in the "heavy microsomes" is higher than that in the "light microsomes", since, as was shown in Figures 18-22, the "light microsomes" contain more ribosomal granules than the "heavy microsomes", and are less rich in membranous vesicles.

SECTION VI

Uptake of radioactive amino acids by "lipid N"

A number of lipid substances capable of incorporating radioactive amino acids have been isolated from a variety of tissues. Barnabei and Ferrari (1961) have demonstrated incorporation of amino acids into a phosphatidopeptide fraction of whole rat liver, and Haining, Fukui and Axelrod (1960) have shown that cell-free preparations of rat liver microsomes and cell sap, incubated separately or together, can also incorporate amino acids into "lipoidal material". Uptake of amino acids by lipids has been observed in other tissues also, e.g. in hen oviduct by Hendler (1959) and in the protoplasts of B. Megaterium by Hunter and Goodsall (1961). These and other studies on the uptake of amino acids by lipid fractions have already been discussed in more detail in the Introduction.

Our next step, therefore, was to discover whether our rat liver "lipid N" was capable of incorporating amino acids in vivo, and to compare its metabolic behaviour with that of the non-lipid protein. The first experiments were carried out on rats fasted overnight after maintenance on a diet containing adequate protein, some of which were given a protein meal 1-1.5 hours before death. The radioactive amino acid (^{14}C -DL-leucine) was injected via the tail vein, and lobes of the liver were removed 5, 10 and 20 minutes after the injection. These time intervals were chosen as those used by Littlefield et al (1955) in their work on rat liver microsomes, since it was hoped to compare our results with their data. Littlefield et al measured amino acid incorporation in vivo into cell sap

and into microsomal fractions prepared by means of deoxycholate, but they studied only the lipid-extracted proteins. In our experiments we examined the activity of both lipid and non-lipid proteins. The results, which are given in Table 27, show that the lipid extract does contain radioactivity. The comparison between fasted and protein-fed animals is best made on the basis of the mean ratio of lipid protein activity to non-lipid protein activity, since the actual specific activities vary greatly from animal to animal according to the amount of injected amino acid actually reaching the liver, and vary also with the degree of isotopic dilution by the amino acid pool. The dilution is, of course, most marked in the protein-fed animals, which were absorbing amino acids at the time of injection. Although the specific activities varied, however, a rough estimation of protein content showed that the actual amount of lipid protein, relative to the amount of non-lipid protein, did not appear to be altered by the feeding of a protein meal 1-1½ hours before death.

Table 27 shows clearly that the feeding of a protein meal to rats fasted overnight markedly increases the uptake of amino acids by the lipid protein relative to the uptake by the non-lipid protein. The effect is seen at all three time intervals tested, and the ratios do not alter greatly within the period 5-20 minutes after injection. Owing to the isotopic dilution difference between the fasted and protein-fed rats already mentioned, it is not possible to say whether the increase in the ratio (sp. act. of lipid protein/sp. act. of non-lipid protein) is due to stimulation of incorporation into the lipid protein or to depression of incorporation

TABLE 27

Incorporation of ^{14}C -leucine into lipid and non-lipid protein of
rat liver

Each experiment was carried out on one fasted and one protein-fed rat. Both rats had been fasted for 18 hours after maintenance on stock diet (exp. 1) or high protein diet with carbohydrate supplement (exps. 2 and 3). The injections were performed 1-1½ hours after the feeding of the protein meal.

The results are expressed as specific activity (cpm/mg. protein). In the case of the non-lipid protein in experiment 1, which was counted as a powder at infinite thickness in an end-window counter, the actual counts have been multiplied by a correction factor (0.8), so that they are equivalent to "cpm/mg protein". The "ratio" represents the sp. act. of the lipid protein sp. act. of the non-lipid protein, and the "mean ratio" is the mean of the values obtained in the three experiments for the "ratio".

Time (min.)	Dietary state	Exp.1		Exp.2		Exp.3		Mean		Ratios			
		Lip.	N.L.	Lip.	N.L.	Lip.	N.L.	Lip.	N.L.	Exp.1	Exp.2	Exp.3	Mean
5	Fasted	3	9	18	54	32	57	18	40	0.33	0.33	0.56	0.41
	Fed	16	26	6	6	80	10	34	14	0.62	1.00	0.80	0.81
10	Fasted	16	30	26	77	26	66	23	57	0.54	0.34	0.40	0.43
	Fed	23	29	13	18	20	25	19	24	0.80	0.72	0.80	0.77
20	Fasted	12	50	65	125	86	109	55	95	0.24	0.52	0.79	0.52
	Fed	47	53	13	26	41	45	38	41	0.89	0.50	0.92	0.77

Lip. = lipid protein sp. act. (cpm/mg. protein)

N.L. = non-lipid protein sp. act.

into the non-lipid protein, but it seems unlikely that the consumption of a protein meal would have the latter effect.

We also have some data on the effects of protein depletion on the response to a protein meal. In two of the above experiments a second group of rats was included which had previously been maintained on a diet deficient in protein. They were similarly fasted overnight and injected with ^{14}C -leucine while still fasting or 1-1½ hours after a protein meal. The results, calculated in the same way as those for the rats maintained on the diet containing adequate protein, are given in Table 28. They show that feeding a protein meal to protein-deficient rats does not produce an increase in the specific activity of the lipid protein relative to that of the non-lipid protein. The ratios of the specific activities of lipid to non-lipid protein on the two diets are shown in Fig. 31, and indicate the contrast in response.

Thus the effect of a protein meal in increasing the relative activity of the lipid protein seems to be confined to animals which, although fasted overnight, are not in a state of protein deficiency. In view of this difference between fasted and protein-fed rats previously maintained on a diet adequate in protein content, we carried out further experiments designed to show whether this difference in activity ratios was confined to certain fractions of the cell or whether it was shared by all the fractions. The experiments were carried out on rats maintained on diets containing adequate protein, in the same way as the experiments described in Table 27, but the liver was taken for analysis at a single time interval only

TABLE 28

Incorporation of ^{14}C -leucine into lipid and non-lipid protein
of protein-deficient rat liver

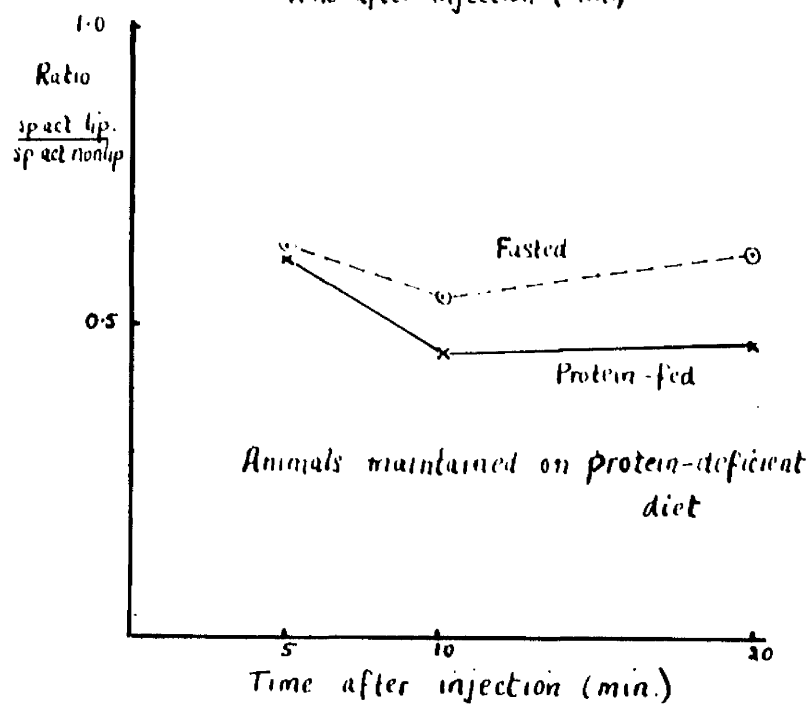
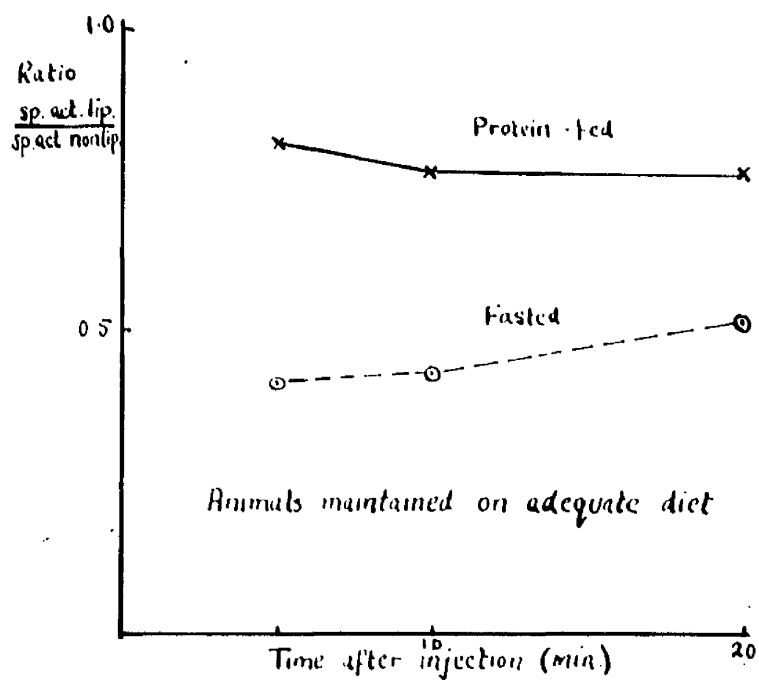
The experiments were carried out on rats which had been kept on protein-deficient diets, with carbohydrate supplement, for 6 days. The experiments were performed, and the results calculated, as in Table 27 (experiments 2 and 3).

Time (min.)	Dietary State	Expt. 2		Expt. 3		Mean		Ratios		
		Lip. N.L.		Lip. N.L.		Lip. N.L.		exp.1	exp.2	mean
5	Fasted	71	130	86	120	79	125	0.55	0.72	0.64
	Fed	22	75	22	23	22	49	0.29	0.95	0.62
10	Fasted	50	140	102	135	76	138	0.36	0.76	0.56
	Fed	19	66	23	35	21	51	0.29	0.64	0.47
20	Fasted	80	192	178	213	129	203	0.42	0.84	0.63
	Fed	27	130	75	100	51	115	0.21	0.75	0.48

Lip. = lipid protein sp. act. (cpm/mg. protein)

N.L. = non-lipid protein sp. act.

FIGURE 31



(5 minutes). The cell fractions were prepared in the usual way, and the results obtained are shown in Table 29. The duplication of some of the figures is poor, but in both experiments all the cytoplasmic fractions show an increase after a protein meal in the activity of the lipid protein, relative to that of the non-lipid protein. Thus the increase is not confined to any particular fraction of the cell. The actual specific activities of the proteins of the various fractions, shown in Table 29, provide a rough check on the efficiency with which our analytical methods remove acid-soluble substances, such as free amino acids. The fairly low specific activity of the cell sap, which at least in the protein-fed rat does not contain large quantities of lipid protein, indicates that the lipid solvent extract of the cell sap is not grossly contaminated by free amino acid. The ratio of lipid protein activity to non-lipid protein activity is rather high in the cell sap compared with the ratio in the other fractions (Table 29), and this may be due to slight contamination. Very little free leucine would be required to raise the specific activity of the small amount of lipid protein in the cell sap. The degree of such contamination must be small, however, since its effect would be more serious in the fasted animal, which has a smaller, and therefore more radioactive, free amino acid pool, and which nevertheless has a lower specific activity in the cell sap lipid protein than has the protein-fed rat. The washing with TCA containing "carrier" leucine must therefore have removed the free amino acids quite efficiently from the acid-precipitated cell sap material. Since the cell sap is the fraction richest in free amino acids, the lipid

TABLE 29

Incorporation of ^{14}C -leucine into lipid and non-lipid proteins
of cell fractions of rat liver

Each experiment was carried out on one fasted and one protein-fed rat, the injections being performed $1\frac{1}{2}$ hours after the protein meal had been given to the fed animals. The results are expressed as specific activities (cpm/mg. protein) in the lipid ("lip.") and non-lipid (N.L.) protein fractions. The ratio of these two figures is given under "R", and the "mean ratio" is the mean of the values for the ratio obtained in the two experiments.

Cell fraction	Dietary State	Experiment 4			Experiment 5			Mean		
		Lip.	N.L.	R	Lip.	N.L.	R	Lip.	N.L.	R
Mitochondria	Fasted	13	13	1.0	36	30	1.2	25	22	1.1
	Fed	25	20	1.25	33	15	2.2	29	18	1.6
Heavy microsomes	Fasted	38	48	0.8	53	44	1.2	47	46	1.0
	Fed	96	83	1.2	70	57	1.2	83	70	1.2
Light microsomes	Fasted	23	34	0.7	58	38	1.5	41	36	1.2
	Fed	92	66	1.4	73	46	1.6	83	56	1.5
Cell sap	Fasted	31	11	2.8	41	18	2.3	36	15	2.3
	Fed	70	19	3.7	58	14	4.1	64	17	3.9

solvent extracts of the other cell fractions may be presumed to be uncontaminated by free radioactive leucine.

The specific activity figures in Table 29 show that the greatest uptake, into both lipid and non-lipid protein, occurs in the microsomes. This is in agreement with the findings of Littlefield et al (1955) and of many other workers. We therefore fractionated our microsomal fractions, using deoxycholate as described by Littlefield et al. In these experiments we prepared our microsomes by Littlefield's method, which gives a preparation roughly equivalent to our "heavy" and "light" microsomes combined. The microsomes, separated into "DOC-soluble" and "DOC-insoluble" fractions, and the cell sap (i.e. the 105,000g supernatant) were treated as usual with TCA and lipid solvents. Table 30 shows the values for the specific activities of the lipid and non-lipid protein of these three fractions obtained in two experiments which were carried out over the time range 5-20 minutes. The duplication between some figures in the two experiments was disappointing, but Figure 32, which shows the average of the specific activities of the various microsome fractions, indicates that, in both fasted and protein-fed animals, the greatest uptake is into the lipid solvent extract of the DOC-soluble fraction, i.e. of the membranous fraction. The lipid-free fraction from the DOC-insoluble material was shown by means of the orcinol reaction to contain 35-45% RNA, and presumably consists of fairly pure ribosomal granules; it is interesting that the specific activity of this fraction is not especially high in either the fasted or the protein-fed rat. The possible significance of this result will be

TABLE 30

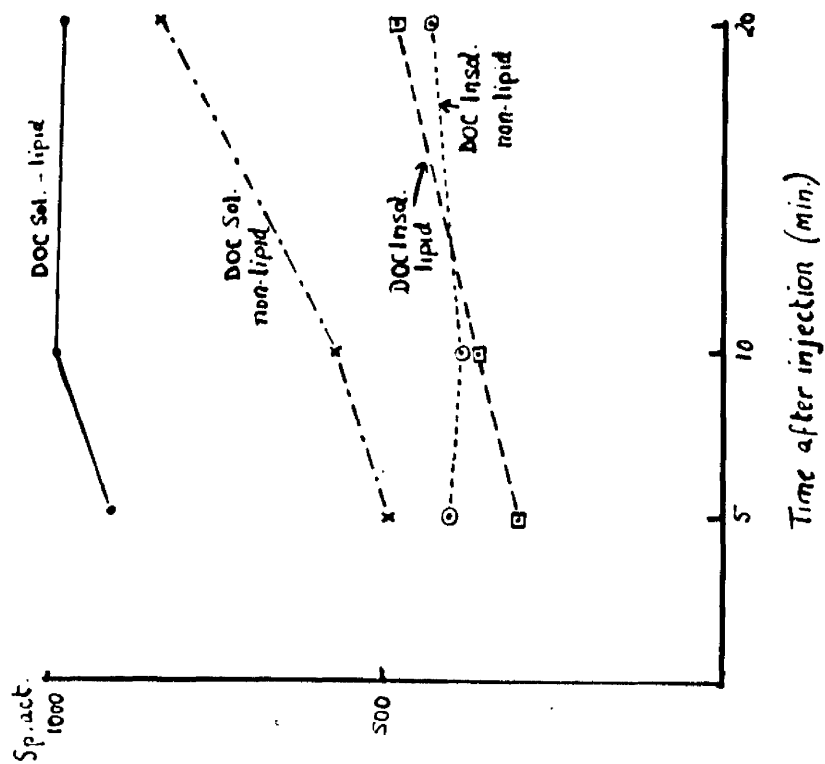
The incorporation of ^{14}C -leucine into the lipid and non-lipid proteins
of DOC-soluble and -insoluble fractions of microsomes

Each experiment was carried out on 1 fasted and fed rat, both previously maintained on an adequate diet. The protein meal was given to the fed animal 1½ hours before the injection. The results are expressed as specific activities and as lipid protein sp.act./non-lipid protein sp. act. (R).

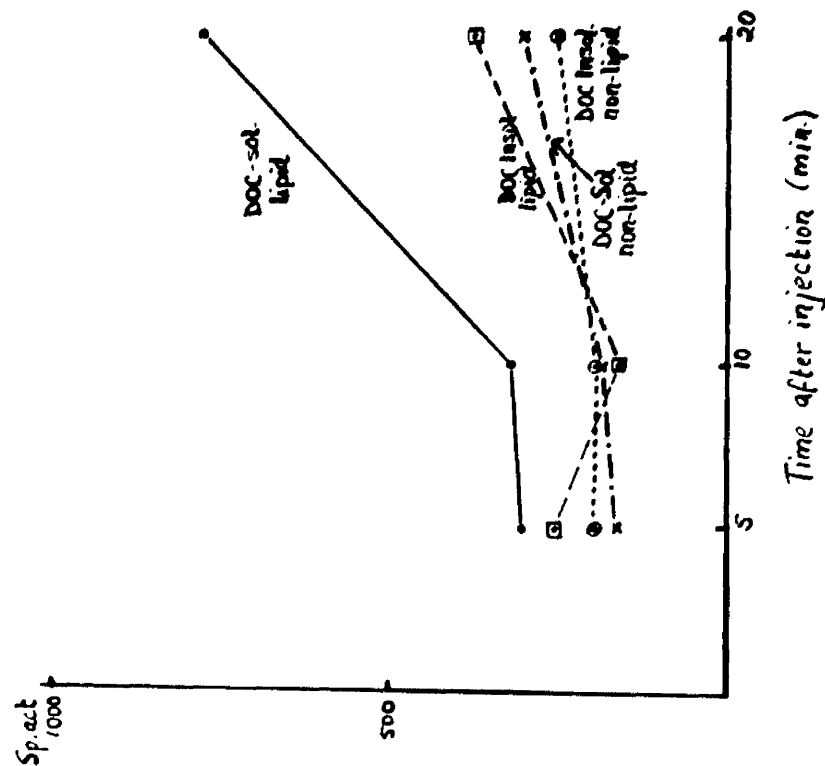
Fract.	Exp.	Result	5 minutes		10 minutes		20 minutes	
			Fasted	Fed	Fasted	Fed	Fasted	Fed
DOC-soluble	6	lip.	950	206	915	159	995	500
		n.l.	743	175	767	152	1065	231
		R	1.27	1.08	1.19	1.05	0.93	1.30
	7	lip.	855	395	1060	475	950	1250
		n.l.	254	153	380	209	611	367
		R	3.37	2.59	2.79	2.26	1.55	3.42
	mean	lip.	903	301	988	517	973	775
		n.l.	498	164	574	180	838	299
		R	2.3	1.8	2.0	1.7	1.2	2.3
DOC-insol.	6	lip.	380	250	449	150	523	375
		n.l.	493	210	527	164	595	159
		R	0.78	1.20	0.85	0.91	0.88	2.36
	7	lip.	221	261	280	167	450	368
		n.l.	312	184	245	200	271	333
		R	0.71	1.42	1.14	0.8	1.66	1.11
	mean	lip.	301	256	365	159	487	372
		n.l.	403	197	386	182	433	246
		R	0.8	1.3	1.0	0.9	1.3	1.7
Cell sap	6	lip.	136	34	218	80	240	57
		n.l.	98	27	120	26	203	27
		R	1.38	1.29	1.82	3.07	1.18	2.10
	7	lip.	216	139	171	90	466	164
		n.l.	94	46	137	60	254	125
		R	2.30	3.01	1.25	1.5	1.84	1.32
	mean	lip.	176	87	195	85	353	116
		n.l.	96	37	129	43	229	76
		R	1.8	2.1	1.5	2.3	1.5	1.7

FIGURE 32

FASTED RAT



PROTEIN-FED RAT



discussed later.

In this series of experiments the activity of the lipid protein relative to that of the non-lipid protein is not, as in earlier experiments, greater in the protein-fed than in the fasted rat; there is, however, some difference between the two, in that the ratio falls steadily from 5 to 20 minutes after the injection in the fasted rat, whereas it rises in the protein-fed rat. At twenty minutes after the injection, the ratio in the DCC-soluble fraction of microsomes from the protein-fed rat is almost twice that in the fasted rat (Table 30).

Since the ratio of lipid protein/non-lipid protein activity was still rising twenty minutes after the injection of radioactive leucine, we next carried out a similar experiment, using deoxycholate fractionation, over a longer time-range. Fasted or protein-fed rats were injected as usual, and killed 30 or 60 minutes later. Table 31 shows the specific activities of the proteins of the cell sap and of the DCC-soluble and DCC-insoluble fraction of the microsomes. In both microsomal fractions the activity of the lipid protein 30 minutes after injection, relative to that of the non-lipid protein, is much higher in the protein-fed rat than in the fasted rat (Table 31). Sixty minutes after injection, however, the ratio in the protein-fed rat has fallen, while that in the fasted rat has risen. Thus in the protein-fed rat the lipid protein of the microsomes appears to reach its highest activity about 20-30 minutes after the injection of radioactive leucine and then to decrease, while the non-lipid protein increases in activity more slowly over a longer period. It would, however,

TABLE 31

Incorporation of ^{14}C -leucine into lipid and non-lipid proteins
of DOC-soluble and -insoluble fractions of rat liver microsomes
at longer times after injection

The experiment was carried out on 2 fasted and 2 protein-fed rats, one of each pair being killed 30 minutes and the other 60 minutes after the injection. The protein-fed rats were given a protein meal $1\frac{1}{2}$ hours before the injection. The results are expressed, as usual, as specific activities and as the ratio of lipid protein sp. act. to non-lipid sp. act.

Fraction	Result	30 minutes		60 minutes	
		fasted	prot.-fed	fasted	prot.-fed
DOC-soluble	lip. pr.	537	796	1040	304
	non-l. pr.	900	415	999	503
	ratio	0.60	1.92	1.04	0.60
DOC-insoluble	lip. pr.	339	360	392	370
	non-l. pr.	431	223	284	461
	ratio	0.79	1.61	1.38	0.80
Cell sap	lip. pr.	273	129	300	268
	non-l. pr.	223	163	350	130
	ratio	1.22	0.79	0.86	1.49

be unwise to place much weight on the detailed results in this experiment, since each figure in the table represents a single specimen.

Another approach to the fractionation of microsomes was made using pyrophosphate and lysolecithin. This experiment was carried out on a rat which happened to be available, and which had been maintained on a diet deficient in protein, although adequate in calories. The liver was removed 20 minutes after the injection of ^{14}C -leucine, and microsomal pellets (105,000g fraction) were prepared and resuspended in sucrose solution. The pellets obtained on further spinning at 105,000g were treated with sucrose-pyrophosphate or with sucrose alone, and then with lysolecithin in sucrose-glycyl-glycine or with the buffered sucrose alone, according to the scheme given in Figure 33. The results of a somewhat similar experiment were discussed in Section V in connection with Table 24, where lysolecithin removed the lipid protein from the pyrophosphate-insoluble fraction of microsomes without affecting the non-lipid protein. As was pointed out in that section, lysolecithin is unpredictable in its effect, and in this experiment both lipid and non-lipid protein have been partially removed by its action, as may be seen from Table 32. Its effect on the radioactivity of the lipid and non-lipid protein of the pellets, however, is much more specific; whether or not the pellet has undergone prior treatment with pyrophosphate, lysolecithin removes about half the amount of lipid protein and 86-89% of the activity of the lipid solvent extract. This implies that the lipid protein removed is highly active, and its specific activity can be calculated to be about 940 c.p.m. per mg. protein.

FIGURE 33

Treatment of microsomal pellets with pyrophosphate
and lysolecithin for experiment described in Table 32

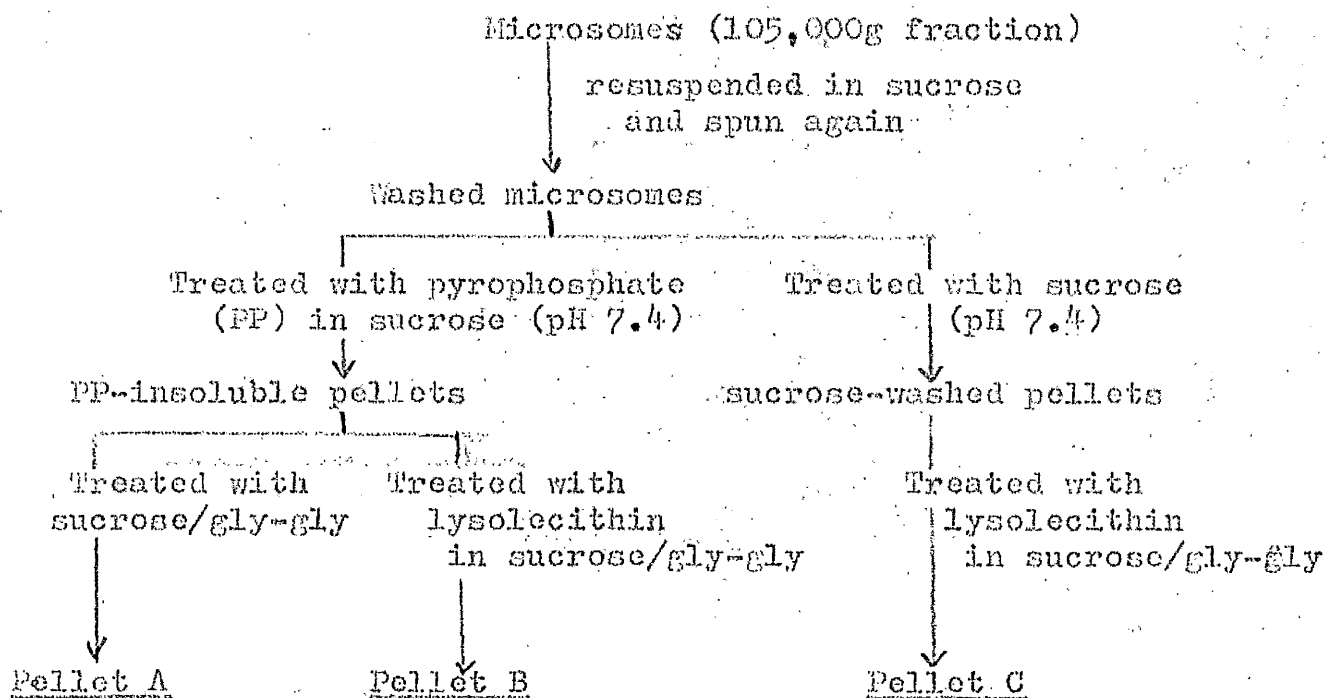


TABLE 32

Fractionation of radioactive microsomesby means of pyrophosphate (PP) and lysolecithin (LL)

The microsomal pellets were treated according to the scheme outlined in Figure 33. The results are expressed (a) as mg. protein in the pellet from 100 mg. liver, (b) as cpm per mg. protein, and (c) as the product of these quantities. By dividing the difference in (c) caused by lysolecithin treatment by the difference in the amount (a), the specific activity (b) of the material removed can be calculated as shown.

Treatment of pellet	Lipid protein			Non-lipid protein		
	amt. (a)	S.A. (b)	(a)x(b)=(c)	amt. (a)	S.A. (b)	(a)x(b)=(c)
A. PP, then buffered sucrose	0.434	515	223	0.615	1560	960
B. PP, then LL	0.222	107	24	0.170	1020	173
C. sucrose, then LL	0.204	154	31	0.169	940	159
Material (X) removed by lysolecithin (calculated):						
X = A - B	0.213	940	199	0.435	1810	787
Ratio X/B	0.96	8.8	--	0.26	1.8	--

(i.e. $\frac{223-24}{0.434-0.221}$). The non-lipid protein, on the other hand, although greatly reduced in amount by the lysolecithin treatment, shows a much less dramatic fall in specific activity. The non-lipid protein removed by lysolecithin proves to have a specific activity of about 1810 ($\frac{960-173}{0.518-0.170}$), that is, less than twice that of the remaining non-lipid protein. Thus lysolecithin preferentially removes a highly active fraction of the lipid protein, of specific activity about 9 times that of the residual lipid protein, whereas the non-lipid protein removed by lysolecithin has a specific activity less than twice that of the remaining non-lipid protein.

Summary:

1. The lipid solvent extract of the TCA-insoluble residue of liver from rats injected 5-60 minutes previously with ^{14}C -DL-leucine contains appreciable amounts of radioactivity. It is unlikely that contamination with free leucine contributes significantly to this activity.
2. The feeding of a protein meal to rats fasted for 18 hours after maintenance on diets containing adequate amounts of protein causes a considerable increase in the specific activity of the lipid protein relative to the specific activity of the non-lipid protein.
3. The feeding of a protein meal to rats fasted after maintenance on a protein-deficient diet does not cause such an increase in the relative activity of the lipid protein.
4. The increase described in paragraph (2) is not confined to any particular fraction of the cell, but has been observed in the mitochondria, in the

"heavy" and "light" microsomes and in the cell sap.

5. At short time-intervals after injection, the greatest uptake of ^{14}C -leucine into both lipid and non-lipid protein occurs in the microsomes.

6. Fractionation of a microsomal preparation (containing both "heavy" and "light" microsomes) with deoxycholate shows that, in both fasted and protein-fed rats, the greatest uptake, 5 - 20 minutes after injection of ^{14}C , is into the lipid protein of the DCC-soluble fraction (membrane).

The DCC-insoluble, non-lipid fraction ("ribosomes"), although it contains some activity, is not so active as the DCC-soluble lipid solvent extract ("membranous lipid protein").

7. In protein-fed animals, the lipid protein of both DCC-soluble and DCC-insoluble fractions seems to reach its maximum activity about 20-30 minutes after injection, and to decrease in activity thereafter. In fasted rats the peak may come a little later, although this is not certain.

8. Lysolecithin removes some of both the lipid and the non-lipid protein from the pyrophosphate-insoluble fraction of microsomes or from whole washed microsomes (in the experiment described in this section). The non-lipid protein removed had a specific activity nearly twice that of the remaining non-lipid protein, while the lipid protein removed had a specific activity about 9 times that of the remaining lipid protein.

There is thus a highly active fraction in the lipid solvent extract which is preferentially solubilised by lysolecithin.

Taken together, these data suggest strongly that the radioactive material

of the lipid solvent extract of microsomes is derived from the membrane, and its sensitivity to variations in dietary conditions recalls the known effect of changes in diet on the endoplasmic reticulum. The finding that uptake of amino acid into lipid protein is stimulated by a protein meal is of particular interest.

DISCUSSION

Chemical nature of the nitrogen in lipid solvent extracts:

This investigation began as a study of the nitrogenous material which is removed by the action of the lipid solvents used to remove lipids from the TCA-precipitated residue of rat liver. It was found that the lipid solvent extract contained a large quantity of nitrogen - over 20% of the total acid-insoluble nitrogen of liver (Table 3). It was shown that very little, if any, of this nitrogen was contributed by nucleic acid (Tables 9 - 11), and phosphorus estimations (Table 3) showed that, even if all the P of the extract were phospholipid P, only about 10% of the solvent-extract N could be accounted for as phospholipid N.

Further investigation showed that the nitrogenous material was soluble in a variety of organic solvents (Tables 5 and 6), although the commonly-used chloroform/methanol/HCl possibly removed some other substance as well (Tables 6 and 8), and was not employed routinely. This wide tolerance in solvents suggests that the material is not identical with some other nitrogenous lipid complexes which have been reported, e.g. a fraction isolated from cytoplasmic membranes of B. Megaterium by Hunter et al (1959), which is soluble in boiling methanol but not in the usual solvents at lower temperatures, or a phosphoprotein isolated from brain by Heald (1961), which is insoluble in chloroform/methanol/HCl. It is possible that the presence of acid is necessary for the extraction of the nitrogenous material by lipid solvents, since none was removed from tissue not treated with TCA (Table 7). The results obtained with microsomes, however, were

variable (Table 8). On one occasion, prior treatment with TCA did not seem to be necessary for the extraction of N by lipid solvents; on another, much less N was extracted from microsomes not treated with TCA than from TCA-treated residues, but an acidified solvent (chloroform/methanol/HCl) was no more efficient than neutral solvents in the extraction of N from the untreated microsomes. Where solubilisation occurs, it does so very rapidly, and longer treatment with the solvent does not increase the efficiency of the process, as may be seen in the case of ethanol in Table 4 and Figure 7. Thus the substance appears to be easily dissolved. Our studies on the N content of fractions prepared by the Schmidt-Thannhauser procedure (Table 12 and Figure 9) gave some indication that it is also fairly easily degraded by treatment with alkali to a form no longer precipitable by acid, since it generally appears to be derived from the acid-soluble supernatant.

Analysis of the amino-N content of an acid hydrolysate (Table 13) and paper chromatography of hydrolysed and unhydrolysed portions (Figures 10 - 14) of solvent extract supported the theory that the "lipid N" consists largely of protein or polypeptide material. It is possible however that, although containing amino acids, the material consists not of protein, but merely of a complex of free amino acids or small peptides with lipid substances. Such a complex would, of course, liberate free amino acids in the conditions used for acid hydrolysis of proteins. It is true that the complex would need to contain an almost complete range of amino acids in proportions similar to those of a typical protein, but this would not be impossible,

and we must consider the question further.

The treatment undergone by the liver homogenate before it is extracted with lipid solvents includes fairly thorough washing with 10% TCA (Figure 4), so that it is unlikely that much acid-soluble material survives to the stage of lipid extraction. It is, however, possible that acid-soluble compounds such as amino acids may be bound to some acid-insoluble material, and so remain in the residue. If the material to which they are bound is soluble in the lipid solvents, the complex may be extracted intact.

In this connection it may be noted that Wren (1960) has drawn attention to the danger of the formation of lipo-amino acid complexes as artefacts. He has shown that, in the presence of protein, free amino acids may form a complex with lipids when exposed to ethanol. In our experiments, however, the free amino acids were removed by the TCA washing procedure before specimens were treated with lipid solvents, so that exposure to ethanol cannot be the reason for the formation of any such complexes which may be present.

We have, moreover, some evidence that the amino acids of the lipid solvent extract are bound in peptide linkage, since our estimation of the peptide content by the biuret reaction (Table 13) indicated that most of the N of the extract could be accounted for as peptide-bound N.

The evidence so far is suggestive of a small protein or polypeptide, and is strengthened by the finding that washing with 0.2 volumes of water, by the procedure of Folch, Lees and Sloane-Stanley (1957), causes the material to separate as a white, phosphorus-free mat at the interphase

(Table 6). This procedure is designed to remove protein from lipid extracts, and suggests strongly that our "lipid N" is indeed protein.

It is well known that if serum albumin is treated with TCA it becomes soluble in ethanol (Levine, 1954; Debro, Farver and Korner, 1957), and the question arises whether the "lipid N" consists of albumin, which is synthesised in the liver. Estimates by Peters (1959) and by Gordon and Humphrey (1961) show that the liver cell contains about 1 mg. albumin per g. wet weight. Since the N content of the lipid extract we have studied would be equivalent to about 30 mg. protein per g. liver, it follows that although albumin may contribute a small part of the solvent-extract N, by far the larger fraction remains unaccounted for.

Our material thus seems to be some peptide or protein other than albumin, but this does not rule out the possibility that it may be in the form of a complex with some lipid substance.

One class of such lipid-peptide complexes is that of the phosphatido-peptides. There are, however, several reasons why our material is unlikely to belong to this category. In the first place, the mat which separated at the interphase on treatment by the procedure of Folch et al (1957) contained most of the nitrogen but very little of the phosphorus of the lipid solvent extract, and it is unlikely that such mild treatment would split off the peptide moiety of the complex. Secondly the phosphatido-peptide prepared from rat liver by Barnabei and Ferrari (1961) is insoluble in acetone, although soluble in acid chloroform/methanol. Thus their material is not identical with ours, although it may contribute towards

the higher figure observed on extraction with acid chloroform/methanol (Tables 6 and 8). Similarly, Ledig, Feigenbaum and Mandel (1961) have reported that rat liver, after extraction with ethanol/ether and methanol/chloroform, still contains phosphopeptides, which they describe as having the characteristics of phosphatidopeptides. Thus phosphatidopeptides are not readily extracted with the lipid solvents we have employed. Finally, Barnabei and Ferrari report that the N/P ratio in their phosphatidopeptide is 2:1. In our experiments (Table 3), the amount of N extracted was 0.52 mg. per 100 mg. liver, and the amount of P was 0.12 mg.; consequently the "lipid N" has too high a N/P ratio to qualify as a phosphatidopeptide. Moreover, the quantity of phosphorus extracted corresponds to the known phospholipid content of liver (Welch, 1945), and little, if any, of it can consist of other phosphorus compounds.

Our chemical investigations into the nature of the "lipid N" suggest, therefore, that it consists of protein or peptide material, probably not containing phosphorus, although this does not rule out the possibility that it exists as a complex with a phosphorus-free lipid. For convenience, it will be referred to as "lipid protein".

Intracellular distribution of "lipid protein":

In many investigations involving analysis of the protein of the liver, attention has been paid only to the lipid-free residue, but it is clear that, if any protein is removed by extraction with lipid solvents, the extract also merits investigation. This applies especially in studies on protein synthesis.

Much of the recent work on protein synthesis has emphasised the importance of the structural components of the cell. We have studied the distribution of the lipid protein within the cell cytoplasm in relation to structure. Its distribution pattern was found to follow that of the phospholipid rather than that of the non-lipid protein, the lipid protein being most plentiful in the membranous fractions of the cell (Section III). Fractionation of the microsomes, which are particularly rich in membranes, by means of reagents which are known to solubilise membranes, confirmed this indication of the membranous origin of the lipid protein. Deoxycholate (Table 23) preferentially removes large quantities of lipid protein from microsomal pellets, along with phospholipid (another membranous component). The non-lipid protein, though not totally unaffected, is solubilised to a lesser extent, and the RNA is little affected. It may be noted in passing that the small amount of RNA which is solubilised by deoxycholate may represent RNA which is actually part of the membrane, rather than of the ribosomal granules. A similar finding was reported by Kuff, Hogeboom and Dalton (1956), and more recently by Chauveau et al (1962). Solubilisation of lipoprotein by deoxycholate was observed in 1952 by Strittmatter and Ball, and our finding (Table 23) that the remaining ("ribosomal") granules are virtually free from lipid protein agrees with the theory that our "lipid N" is a membranous lipoprotein.

The converse approach, the solubilisation of the ribosomal granules without the membranes, can be made by means of pyrophosphate. The data

we obtained in this way (Table 21) also accord with the theory of the membranous nature of the lipid protein. The RNA and non-lipid protein are diminished by pyrophosphate treatment, while the lipid protein and phospholipid, again acting in parallel, are partially solubilised. It is of interest that about 30% of the RNA is not solubilised by pyrophosphate, and that it differs in composition from the 70% which is solubilised. In this laboratory, Goswami, Barr and Munro (1962) have shown that the smaller fraction, presumably RNA associated with the membrane, contains no pseudouridylic acid, whereas the pyrophosphate-soluble fraction contains 2.2% of pseudouridylic acid. This finding confirms that the differential action of pyrophosphate is due to its specific effect on a subcellular structure, rather than to random attack on all the microsomal RNA and non-lipid protein.

Our other attempts at differential solubilisation of microsomal preparations, using lysolecithin (Table 24) and a bacterial toxin (Tables 25 and 26), gave less clear-cut results, and the effects of these reagents are less well documented in the literature than are those of deoxycholate and pyrophosphate. Nevertheless, the parallel behaviour of lipid protein and phospholipid on the one hand, and of RNA and non-lipid protein on the other, were observed in all cases. Reagents which attack the membranous phospholipid also attack the lipid protein, and those which attack the ribosomal granules have no effect on the lipid protein. Only in the case of the experiment with haemolysin (Table 26) is there an anomalous result, in that whereas the other components are solubilised,

if at all, only by the active toxin, the lipid protein appears to be solubilised simply by the conditions of incubation in Krebs-Ringer bicarbonate solution at 37° for 1 or 2 hours. In this it differs from the phospholipid, which is undissolved; on the other hand, even if the membrane disintegrated completely in such conditions, the phospholipid fragments would not be expected to dissolve in the aqueous solution, whereas the lipid protein (or a peptide or protein moiety derived from a lipid protein complex) might well dissolve more readily. Another form of treatment which might give interesting results in microsome fractionation is incubation with RNase, and it is hoped to carry out such an experiment later.

In view of the similar conclusions drawn from four different methods of chemical subfractionation of microsomes, the weight of evidence seemed to suggest the cytoplasmic membranes as the most likely source of the lipid protein. Comparison of the amounts of protein, RNA and phospholipid in the two mechanically-separated microsomal fractions ("heavy" and "light") showed that the percentage of lipid protein and phospholipid in the "heavy microsomes" was about twice that in the "light microsomes" (Table 15). The implication, that the "heavy microsomes" were richer in membranous vesicles than the "light microsomes", was confirmed by electron microscopy of the two fractions (Figures 19 and 21). Thus we have yet another piece of evidence that the cytoplasmic membranes are the source of the lipid protein.

The endoplasmic reticulum of the liver cell is of two types - rough and smooth, the former being much more plentiful. The smooth reticulum consists of membranes without ribosomal granules, but probably containing

a small amount, perhaps 5%, of RNA (Chauveau et al, 1962). The rough endoplasmic reticulum consists of vesicles studded with small dense ribonucleoprotein granules, the ribosomes. Chauveau et al have recently shown that the membranes and granules can be separated, without detergent treatment, by ultracentrifugation, the membranes separating as a pellicle. The pellicle contains all the phospholipid of the preparation, and a number of enzymes characteristic of the membrane. It would be interesting to see whether it also contains all the lipid protein.

Effect of diet on the amount of lipid protein:

It has been shown by Fawcett (1955) and by Bernhard and Rouiller (1956) that the reticulum responds to variations in diet. Both groups of workers found that in rats starved for 5 or 6 days the reticulum decreased in quantity, and that regrowth could be seen within 24 hours of the feeding of a protein meal. The early stages of regrowth could be seen as little as 6 hours after the meal. A carbohydrate meal did not produce regrowth. It should perhaps be said that the appearance of liver cells in the electron microscope varies greatly, and that even adjacent cells may differ considerably, so that such descriptions as "diminished amount of reticulum" and "regrowth of reticulum" reflect the general impression given by a number of sections. The problem is made worse by the fact that the area seen in the electron microscope is so small, and Fawcett has said (personal communication) that he would like to repeat his work published in 1955 using more modern techniques. We shall, however, assume that, at least in outline, the results published then are valid,

since our own electron micrographs tend to confirm them, and they fit in well with our analytical data.

Results obtained by Munro and Clark (1960) suggest that the breakdown of endoplasmic reticulum caused by protein depletion (Laird, Barton and Nygaard, 1955) begins during the first day of feeding a protein-deficient diet. Our data on the composition of the microsomes and cell sap of rats fasted for 18 hours (Table 20) indicate that the feeding of a meal of casein to such fasted rats may lead to a slight but consistent increase in the lipid protein content of the microsomes, and a corresponding decrease in that of the cell sap. Since the products of breakdown of the reticulum during fasting are probably to be found in the cell sap, this finding suggests a connection between the lipid protein and the endoplasmic reticulum.

Further experiments were carried out on the effect of protein administration (Table 20) in which the conditions used were more closely comparable with those used by Fawcett and by Bernhard and Rouiller. In these, the rats were starved for 5 days, and killed either fasting or after a protein meal. In one case the liver was removed $1\frac{1}{2}$ hours after the protein meal, and in the other case 6 hours after the meal. Comparison of the lipid protein content of the microsomes from the liver of the protein-fed rats with the content in the fasted rat shows that at both time intervals the protein meal has caused an increase in microsomal lipid protein. The liver of the rat killed 6 hours after the meal was examined in the electron microscope (Figure 24), and shows signs of regeneration of endoplasmic reticulum, while the fasted rat (Figure 23) appears to have less than the

normal amount of reticulum. Thus once again changes in the lipid protein content seem to reflect changes in the amount of endoplasmic reticulum.

The next step was to subdivide the microsome fraction by chemical disintegration, in order to see whether the effects of protein feeding on the lipid protein were confined to the membrane fraction. If the lipid protein is indeed a component of the reticulum, fluctuating in amount with dietary conditions, and if it is separable from the lipid protein of the microsomes by chemical treatment, it ought to be possible to obtain a dietary effect which is confined to the appropriate chemical fraction of the microsomes. An experiment of this type was performed, using pyrophosphate to fractionate the microsomes of rats which had been starved for 5 days and killed either fasting or 2 hours after a protein meal. As was anticipated, the increase in microsomal protein produced by the protein meal (Table 22) is confined to the pyrophosphate-insoluble residue, i.e. the membranous fraction. The soluble fraction (the ribosomal granules) shows no increase. Thus the lipid protein represents a chemically and metabolically distinct fraction of the microsomes.

Evidence obtained by Munro and Clark (1960) on the effect of a protein-deficient diet on the metabolism of RNA in the liver suggests that loss of endoplasmic reticulum may be produced by protein deficiency as well as by complete starvation. We therefore carried out a series of experiments (Tables 18 and 19) in which rats were maintained for 11 days on diets of different protein and calorie contents, six diets in all. Although we obtained evidence that protein depletion over such a period, especially

when combined with calorie deficiency, not surprisingly leads to a fall in the amount of all the constituents estimated in the liver, the percentage composition of the liver cell and of the individual cell fractions does not appear to change. That is, although the amount of lipid protein is diminished during such protein depletion, the non-lipid protein and other cell constituents are reduced in proportion; there is no specific loss of any of the constituents estimated. An exception is DNA, which remains constant, since, presumably, the number of cells in the liver is not reduced. It follows that, if there is a reduction in the microsomes during protein depletion, the complete structural unit is lost, and not merely a fraction. This agrees with the observation that there is little difference in the appearance (as distinct from the amount) of the rough endoplasmic reticulum (Figures 16 and 17), or of the microsomal pellets (Figures 19 - 22), in adequately-fed and protein-deficient rats. This finding may be compared with those of Table 20, which showed that the lipid protein, and possibly the phospholipid, were specifically increased during the regeneration of endoplasmic reticulum caused by the feeding of a protein meal to starved animals. The reason for the difference between this specific effect and the non-specific effect of the loss of reticulum is probably that the regenerating reticulum consists initially of membranes without granules, as was shown by Fawcett (1955). It would therefore be expected to be especially rich in phospholipid, and in lipid protein if this is a membranous component. When reticulum disintegrates, however, all components will be lost simultaneously.

In view of these indications that the amount of lipid protein varies with changes in the endoplasmic reticulum, it would be of interest to study the relationship between lipid protein and the reticulum in malignant cells. Certain types of fast-growing hepatoma tissue have been shown to lack reticulum (Howatson and Ham, 1955; Novikoff, 1957), and it would be interesting to investigate their content of lipid protein.

Metabolic role of the lipid protein:

Finally, we considered the possible metabolic role of our lipid protein, particularly in protein synthesis. The idea that the microsomal membrane may play a part in protein synthesis is not a new one, and the Introduction contained a discussion of some of the evidence for its role in the synthesis and secretion of proteins. There is also a large body of evidence of the capacity of lipids to form complexes with amino acids, and this also was discussed earlier.

Our own evidence shows, firstly, that there is a considerable uptake of intravenously injected radioactive leucine into the lipid solvent extract of liver over the time range 5-60 minutes after injection (Tables 27 and 31). Since the lipid solvents are used immediately after the third acid wash with TCA, we must consider the possibility that the lipid solvent extract is simply contaminated with free radioactive amino acid. The amount of free amino acid cannot be very great, since paper chromatography of the unhydrolysed extract (Figures 11 and 14) failed to detect any trace of even the most abundant amino acids. Although paper chromatography is fairly sensitive, however, radioactivity measurements are more sensitive

still, and a very small amount of free leucine present in the extract could lead to large errors. For reasons discussed in Section VI, however, it is unlikely that there is serious contamination of the extract.

It has not yet been shown that the radioactive leucine in the lipid extract is actually bound to the "lipid protein". Attempts to fractionate the lipid solvent extract by paper electrophoresis were unsuccessful. They did, however, furnish more evidence that the activity of the extract is not due to contaminating amino acid, since almost all of the radioactivity remained at the origin in conditions in which free amino acids would move on the paper. Possibly the application of the Folch water-washing procedure for the separation of lipids and protein would yield further information on the binding of the ^{14}C -leucine.

We had already shown that lipid protein was influenced by variations in diet, and now proceeded to study the effect of such variations on the uptake of ^{14}C -leucine by the lipid solvent extract of whole liver. Our experiments on rats fasted overnight after maintenance on adequate or protein-deficient diets for 6 days indicated that the uptake of leucine by the extract was sensitive both to long-term dietary conditions and to short-term fasting and re-feeding with protein. Adequately nourished rats, which presumably begin to lose endoplasmic reticulum during the 18 hours' fast, show a stimulation of uptake into the lipid fraction 2 hours after a protein meal (Table 27) which no doubt reflects a re-synthesis of reticulum. Rats depleted of protein (Table 28), however, do not show such a response to a protein meal. The lipid extract of the protein-

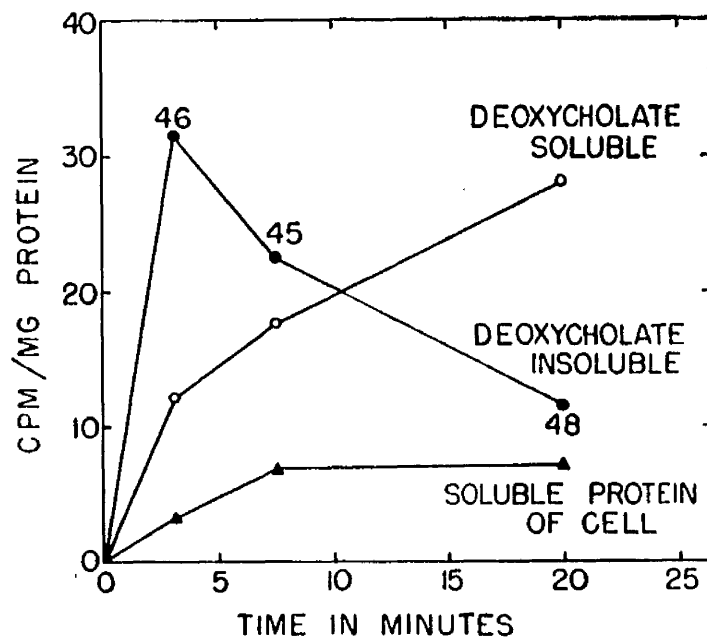
deficient rats does take up ^{14}C -leucine, but there appears to be no difference between the fasted and the protein-fed animals in the uptake in the solvent extract relative to that in the non-lipid protein. It may be that during prolonged protein depletion the endoplasmic reticulum is reduced or modified so that it cannot immediately respond to a protein meal in the manner of the normal rat. Alternatively, the process whereby the nucleus controls the assembly of amino acids (e.g. the production of "messenger RNA") may be disturbed. The appearance of the nucleolus is greatly altered during protein deficiency.

In these experiments we have shown a specific stimulation of amino acid uptake in the lipid fraction in response to a protein meal. This is of particular interest since the addition of amino acids to the medium does not stimulate the uptake of radioactive amino acid in the non-lipid protein of liver tissue slices (Munro, personal communication), and in effect the feeding of a protein meal is equivalent to the addition of amino acids. The finding that there is a specific increase in the uptake of amino acid into the lipid fraction suggests that the membranes of the cell may be an important factor in the process of protein synthesis. Whether or not membrane-free ribosomes (prepared by deoxycholate treatment) can incorporate amino acids is not certain, but evidence obtained by Korner (1959) suggests that they can, although not so much as the intact microsomes. Palade and Siekevitz (1956) earlier were unable to obtain any incorporation with such particles. Thus the ability of isolated ribosomal granules, of the liver at least, to incorporate amino acids is

less than that of the whole (granules + membrane) fraction. Our own evidence obtained from fractionation experiments with deoxycholate or with lysolecithin suggests that the uptake is actually greatest in the membranous fraction at the time intervals studied. If the ribosomes are the primary binding site in the microsomes of amino acids (or sRNA-bound amino acids), they would be expected to have very high activity. It is true that a large part of the ribosomal protein is probably structural, and may serve to dilute the activity of a small proportion which is active in binding amino acids. Nevertheless, as was shown in Table 27, the DOC-insoluble fraction of the microsomes contains only about 27% of the total protein (lipid and non-lipid) of the microsomes; presumably the DOC-soluble (membranous) fraction, which contains the remaining 63%, is also liable to contain a proportion of metabolically inert protein, yet its specific activity is greater than that of the DOC-insoluble fraction (Table 30).

Our time intervals in Table 30 are slightly different from those investigated by Littlefield et al (1955), who measured the amino acid uptake during the first few minutes after injection as well as 20 minutes later. Their results are shown in Figure 36, and may be compared with our own data obtained by the use of deoxycholate (Table 30 and Figure 32). Littlefield et al extracted their acid-precipitated microsomes with lipid solvents, and measured the radioactivity of the lipid-free fraction only. Over the time range in which comparison is possible (10-20 minutes), their findings are in substantial agreement with our own data for non-lipid protein. The specific activity of the DOC-soluble fraction increases,

FIGURE 36



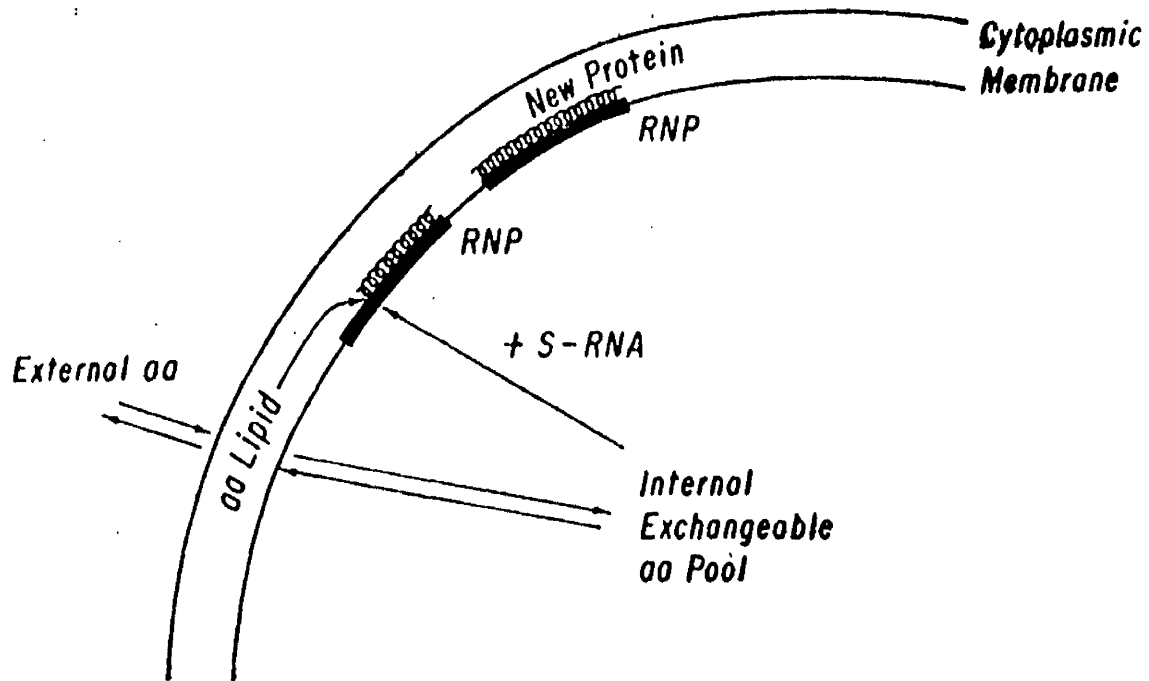
Incorporation *in vivo* of a small dose of leucine- C^{14} into the two components of the microsomes and into the soluble protein of the cell. 0.16 μmole of DL-leucine- C^{14} , 1.8×10^7 c.p.m. per mg., in 0.5 ml. of isotonic saline was injected intravenously at zero time into a 270 gm. rat. The per cent RNA by weight of each deoxycholate-insoluble sample is indicated. The per cent RNA averaged 2.1 in the deoxycholate-soluble fractions of the microsomes and 1.7 in the soluble fractions of the cell.

while that of the DOC-insoluble fraction falls, or rises more slowly. By taking 5 minutes as the first time interval, we have missed the maximal activity in the DOC-insoluble fraction. In both Littlefield's experiments and ours, the specific activity of the protein of the DOC-soluble (membrane) fraction 10 minutes after injection equals that of the DOC-insoluble (ribosome) fraction. Before this time, the data of Littlefield et al show that the ribosome protein exhibits greater activity; thus at 3 minutes its activity is 1.56 times that of the non-lipid protein of the membrane at 10 minutes. Our own data, however, show that the activity of the lipid protein of the membrane 5 minutes after injection is 1.66 times that of the non-lipid protein of the membrane at 10 minutes. Thus, even at early time-intervals (3 minutes), when the specific activity of the DOC-insoluble non-lipid protein in Littlefield's experiments is at its maximum, its activity does not exceed that of the DOC-soluble lipid protein.

Since our first time interval (5 minutes) was later than that used by Littlefield et al (2 or 3 minutes) it is possible that the fraction showing the highest activity at 5 minutes in our experiments (the lipid extract of the DOC-soluble fraction of microsomes) does not represent the original site of amino acid uptake. It may, however, contain a precursor of the active material in the non-lipid fraction, since the activity of the latter tends to rise, while the lipid fraction activity falls. Study of the activity of the various fractions during the first few minutes might throw more light on the relationships among them.

Finally, it is interesting to consider in the light of these findings

FIGURE 37



(1) Protein is synthesized at an interface between a lipo-protein membrane and a ribonucleoprotein film. (2) Amino-acid preferentially approaches the interface from the lipoprotein membrane side, either from the outside or inside of the cell. (3) Amino-acid from the interior pool could approach from the RNA side utilizing *S*-RNA reactions. RNP = ribonucleoprotein or ribosome; *S*-RNA = soluble ribonucleic acid. Cytoplasmic membrane is to be representative of any cell membrane such as endoplasmic reticulum or nuclear membrane

(Hendler, 1962)

a scheme recently proposed by Hendler (1962) providing a role for the membrane in the synthesis of proteins. A diagram of this scheme is shown in Figure 37. Some such mechanism might explain the high uptake we have observed in the lipid fraction of the microsomal membrane, as well as the widely-recognised uptake of amino acids by the ribosomes. As was pointed out in the Introduction, there may be more than one way of transferring amino acids to the ribosomes.

SUMMARY

Chemical nature of the nitrogen in lipid solvent extracts:

1. It has been shown that treatment of TCA-precipitated rat or mouse liver residue with certain solvents used to extract lipids leads to the solubilisation of more than 20% of its total acid-insoluble nitrogen. Only about 10% of this N can be accounted for as phospholipid N.
2. The nitrogenous material ("lipid N") is soluble in a variety of solvents, e.g. acetone or ethanol at 0°, and ethanol/chloroform or chloroform/methanol/HCl (200:100:1) at room temperature. The last-mentioned solvent possibly dissolves more material than the others, but it is not known if the excess is different in nature. The presence of acid may be a factor in the extraction of nitrogenous material. Longer times of exposure to the action of ethanol at 0° (up to 24 hours) do not lead to greater losses.
3. It was shown that the "lipid N" contains negligible amounts of nucleic acid.
4. The material appears to be more susceptible to the action of 1N alkali at 37° than the "average" protein of the liver cell. Such treatment for 16 hours generally reduces it to fragments no longer precipitable by 10% TCA at 0°.
5. Estimations by the ninhydrin reaction of the amino nitrogen content of a hydrolysate of the solvent extract indicates that almost all the nitrogen of the extract can be accounted for as amino nitrogen.
6. Paper chromatography of the hydrolysate reveals that the extract

contains a wide range of amino acids, probably sufficient in quantity to account for all the solvent nitrogen, and having the appearance of the amino acid content of a typical protein hydrolysate. A chromatogram of an unhydrolysed sample was blank except for one small faint spot, probably peptide.

7. Estimation of the unhydrolysed extract by a quantitative form of the Biuret reaction showed that nearly all its nitrogen is peptide-bound.

8. Washing the extracted material, dissolved in chloroform/methanol, with 0.2 volumes of water according to the procedure recommended by Folch, Lees and Sloane-Stanley (1957) for the separation of proteins and lipids, leads to the separation of a white mat at the interphase. The mat contains almost all the non-phospholipid nitrogen of the extract, but the phosphorus remains in the organic phase.

The material is therefore likely to be a non-phosphorus-containing protein, but is probably not albumin.

Intracellular distribution:

9. Cell fractionation studies show that all four cytoplasmic fractions investigated (mitochondria, "heavy" and "light" microsomes, and cell sap) contain some "lipid protein", but that its distribution is not uniform. The mitochondria and microsomes contain more than the cell sap, and the "heavy microsomes" more than the "light microsomes". These are the membranous fractions, and it has been shown by electron microscopy that the "heavy microsomes" are richer in membranous vesicles than the "light microsomes". The distribution of "lipid protein" follows that of the

phospholipid rather than that of the non-lipid protein.

The "lipid protein" may therefore be a component of the cytoplasmic membranes or endoplasmic reticulum.

Effect of diet:

10. Variations in the level of protein or calorie intake over 11 days did not produce any specific decrease in the amount of "lipid protein" of the whole cell, or of any cytoplasmic fraction in growing rats. Protein deficiency did lead to a decrease in all the cell constituents estimated, except DNA, but the percentage composition was unaltered. Electron microscope studies of the livers of these rats showed that the endoplasmic reticulum did not seem to be altered in appearance. If the endoplasmic reticulum is decreased by protein depletion, the structural units must be lost as a whole, i.e. membranes plus granules.

11. Rats given a meal of protein after fasting overnight appeared to have slightly more lipid protein in the microsomes, and slightly less in the cell sap, than rats also fasted but not given a protein meal. During fasting the endoplasmic reticulum breaks down, and a protein meal reverses this process. Thus it appears that the lipid protein of the microsomes may be part of the reticulum, and that of the cell sap may be breakdown products of the reticulum.

12. A similar increase in the lipid protein content of the microsomes was noted 1½ and 6 hours after a protein meal had been given to rats starved for 5 days. Six hours after the meal, there appeared to be larger amounts of endoplasmic reticulum in the liver than in the liver of the rat also

starved but not given a protein meal.

All these findings suggested a relationship between the lipid protein and the endoplasmic reticulum.

Chemical fractionation of microsomes:

13. Fractionation of microsomal pellets by means of pyrophosphate, which solubilises ribosomal granules while leaving the membranes intact, reduced the RNA and non-lipid protein content of the microsomes without affecting their lipid protein or phospholipid content.

14. This pyrophosphate fractionation technique was applied to microsomes from rats starved for 5 days as in (12) and killed either fasting or 2 hours after a protein meal. It was found that the increase in lipid protein produced by the protein meal was confined to the pyrophosphate-insoluble fraction of the microsomes.

15. The converse treatment with deoxycholate, which removes the membranes but not the ribosomal granules, led to preferential solubilisation of the lipid protein, although some non-lipid protein was also removed. The residual "ribosomes" were apparently fairly pure, since they contained 43% of RNA, and contained very little lipid protein.

16. Lysolecithin was less satisfactory as a means of fractionating microsomes, as it was inconsistent in its effect on the non-lipid protein. On one occasion it did remove some lipid protein from a pyrophosphate-insoluble residue of microsomes, without affecting the non-lipid protein.

17. A toxin prepared from Staph. aureus which was known to be capable of lysing red cells was tested for its effect on microsomes. It was inactive

in the presence of sucrose. In bicarbonate buffer it partially solubilised the non-membranous components of the microsomes, the RNA and non-lipid protein, but not the phospholipid. It did remove some of the lipid protein, but it was discovered that the lipid protein content of the pellet fell just as much during incubation with inactivated toxin, or even with the incubation medium alone. The lipid protein thus seemed to be partially solubilised by incubation at 37° in bicarbonate buffer. It is not known whether this was due to chemical breakdown of the complex, but it seems more likely that the membrane disintegrated as a whole. The phospholipid would not be expected to dissolve in the aqueous medium.

Thus the results obtained by chemical fractionation of microsomes also suggest that the lipid protein is a component of the microsomal membrane.

Metabolic role of lipid protein:

18. Studies carried out on rats injected intravenously with ^{14}C -DL-leucine showed that the lipid solvent extract of the liver from 5-60 minutes later contained considerable amounts of radioactivity, which were probably not due to contamination with free amino acid.

19. The uptake of radio-leucine in the lipid solvent extract, relative to that in the non-lipid protein, was stimulated in rats fasted overnight by the feeding of a protein meal.

20. This stimulating effect of a protein meal was not observed in rats which had been depleted of protein by maintenance on a protein-free diet for 6 days. These rats showed signs of protein deficiency, such as a

greatly enlarged nucleolus, and this may reflect a disturbance in the mechanism controlling the production of RNA. Although even longer periods of protein deficiency did not produce any very marked change in the appearance of the endoplasmic reticulum, it may nonetheless have been modified by protein depletion, so that it is no longer able to respond to the protein meal.

21. In adequately nourished rats, the increase in the activity of the lipid solvent extract relative to that of the non-lipid protein, which occurs in response to a protein meal, was seen in all the cytoplasmic fractions.

22. The uptake of ^{14}C -leucine was highest in the microsomes, and was particularly high in the lipid solvent extract of the DCC-soluble fraction (i.e. of the membrane). The activity reached its highest level about 20-30 minutes after the injection of the leucine.

23. Further evidence that the lipid extract of the membrane is highly labelled comes from an experiment in which a microsomal pellet, freed of some of its non-lipid protein and RNA by pyrophosphate treatment, was then treated with lysolecithin. The lysolecithin removed some of both the lipid protein and the non-lipid protein, but the ratio of the activity of the material removed to that of the material remaining in the pellet was very much higher in the lipid protein than in the non-lipid protein.

Our evidence therefore suggests that the non-phospholipid nitrogenous material removed by a number of organic solvents from a TCA-treated residue of liver is probably protein or polypeptide in nature and contains

no phosphorus, that it is derived from the endoplasmic reticulum, that it is specifically sensitive to starvation and to re-feeding with protein, and that it is associated with a highly radioactive fraction in the microsomal membrane from 5-30 minutes after intravenous injection of ^{14}C -leucine.

APPENDIX

Some aspects of the analysis of nucleic acids in liver

In part IIA of the "Results" section, it was noted that estimates for the DNA content of liver obtained by the Schneider ICA extraction method were much lower than those obtained by the Schmidt-Thannhauser alkaline digestion method (see Table 11). In view of this apparent inefficiency of ICA as a DNA extractant, in the conditions we had used, we carried out a study on the relative efficiency of ICA extraction of nucleic acids at different concentrations and temperatures, the results of which are given in Table 33 and in Figure 38. Values obtained by the Schmidt-Thannhauser procedure were used as a standard of reference. Since the acid-soluble fraction of the Schmidt-Thannhauser digest includes phosphorus-containing contaminants (Hutchison et al., 1956), its P content is slightly higher than the RNA-P value given by the orcinol reaction. The P content of the acid-insoluble residue agrees well with the DNA-P figure obtained by the Ceriotti method. By comparison with these Schmidt-Thannhauser values, the recovery of RNA by ICA extraction at different concentrations and temperatures is quite efficient; indeed, the values at higher temperatures are too high, presumably because of degradation and extraction of orcinol-reacting material other than RNA. Recovery of DNA, on the other hand, was considerably lower by the ICA extraction method, even the most favourable conditions (1.6N ICA at 70°C) giving a yield only 87% of that obtained by the Schmidt-Thannhauser procedure.

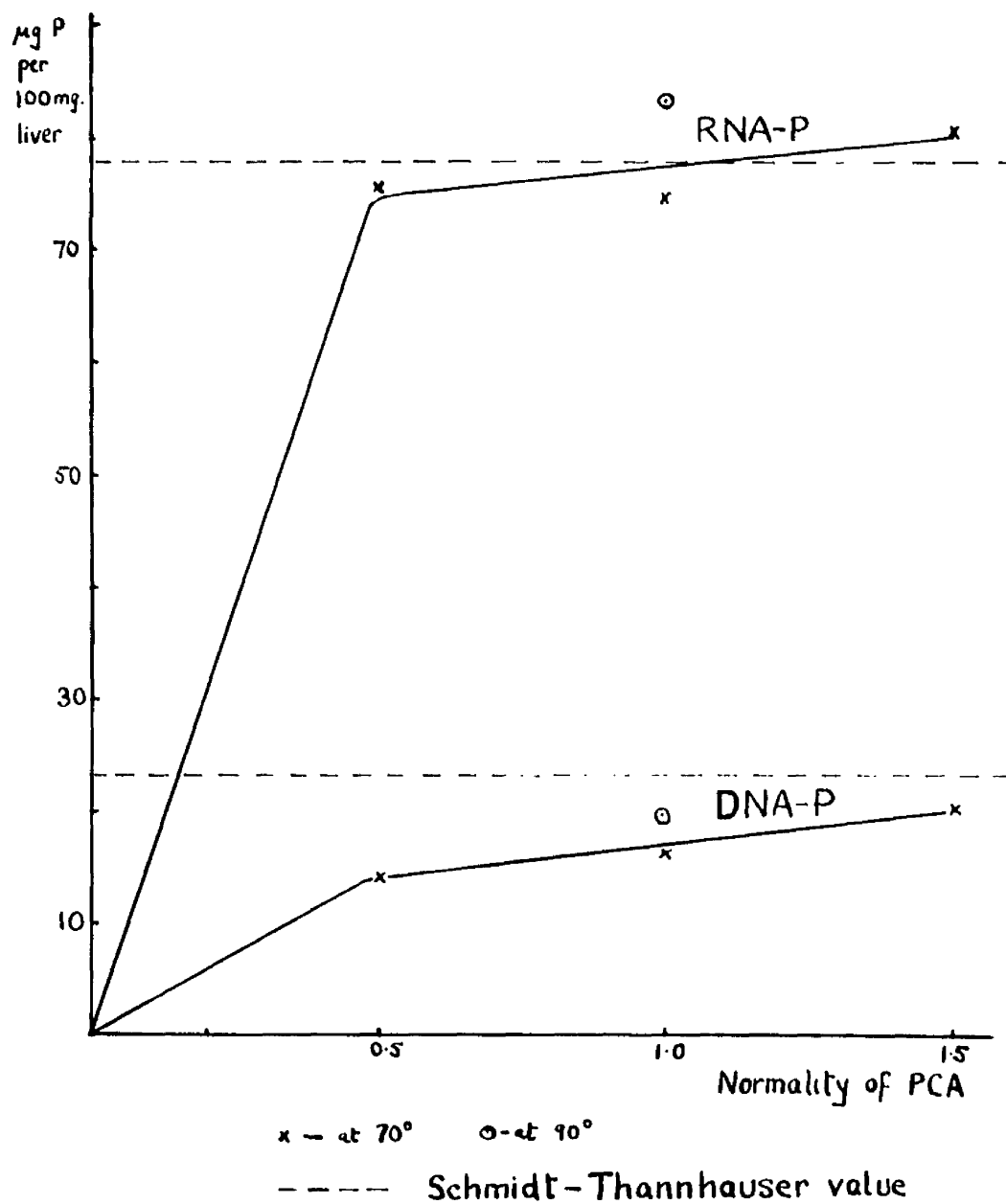
TABLE 33

Recovery of nucleic acids from rat liver under various conditions
of FCA extractions

Two 20-minute extractions were carried out. The orcinol and Ceriotti procedures were used to measure the nucleic acids of both the FCA extract and the Schmidt-Thannhauser fractions. The results are the mean data obtained in two experiments (one of which was carried out by Dr. W. C. Hutchison), and are given as $\mu\text{g. nucleic acid P per } 100 \text{ mg. liver}$. The figures in brackets are the recoveries by FCA extraction relative to those by the Schmidt-Thannhauser procedure.

Treatment		Total P	RNA P	DNA P
Schmidt-Thannhauser	RNA fraction	85.2	78.2	11.4
	DNA fraction	23.1	-	23.3
FCA extraction	0.5N at 70°C	90.8	75.8 (97%)	14.3 (31%)
	1.0N at 70°C	88.4	74.9 (96%)	16.5 (71%)
	1.6N at 70°C	95.2	81.0 (103%)	20.3 (87%)
	1.0N at 90°C	109	83.6 (107%)	19.9 (85%)

FIGURE 38



Efficiency of PCA extraction of nucleic acids

The results of a more extensive examination of the effects of temperature and of concentration of PCA on the extraction of nucleic acids from rat liver are given in Figure 39*. Two temperatures commonly used for PCA extraction, 70°C and 90°C, were examined in conjunction with a wide range of PCA concentrations. At 70°, extraction with concentrations of PCA of 0.8N or less resulted in DNA values which were much lower than those obtained by the Schmidt-Thannhauser method. Within the range 1N to 3N PCA, the values were only a little less than the Schmidt-Thannhauser figures; at higher concentrations the values again fell off, presumably owing to the destruction of deoxypentose.

At 90°C, this destruction begins at much lower concentrations of acid, and the values never exceed 90% of the Schmidt-Thannhauser series.

The RNA is completely extracted at 70°C by 0.5N PCA, and higher concentrations remove further quantities of orcinol-reacting material, so that the values eventually exceed those obtained by the Schmidt-Thannhauser method. A similar increase occurs when the extraction is performed at 90°C. Thus PCA at too high a temperature or concentration is liable to extract orcinol-reacting substances other than RNA.

It appears, therefore, that while the PCA extraction method may be satisfactory for estimation of RNA provided the concentration of PCA and the temperature of extraction are not too high, it is not a suitable

* The analysis of some of the liver specimens was carried out by Dr. W. C. Hutchison. Similar results were obtained using spleen.

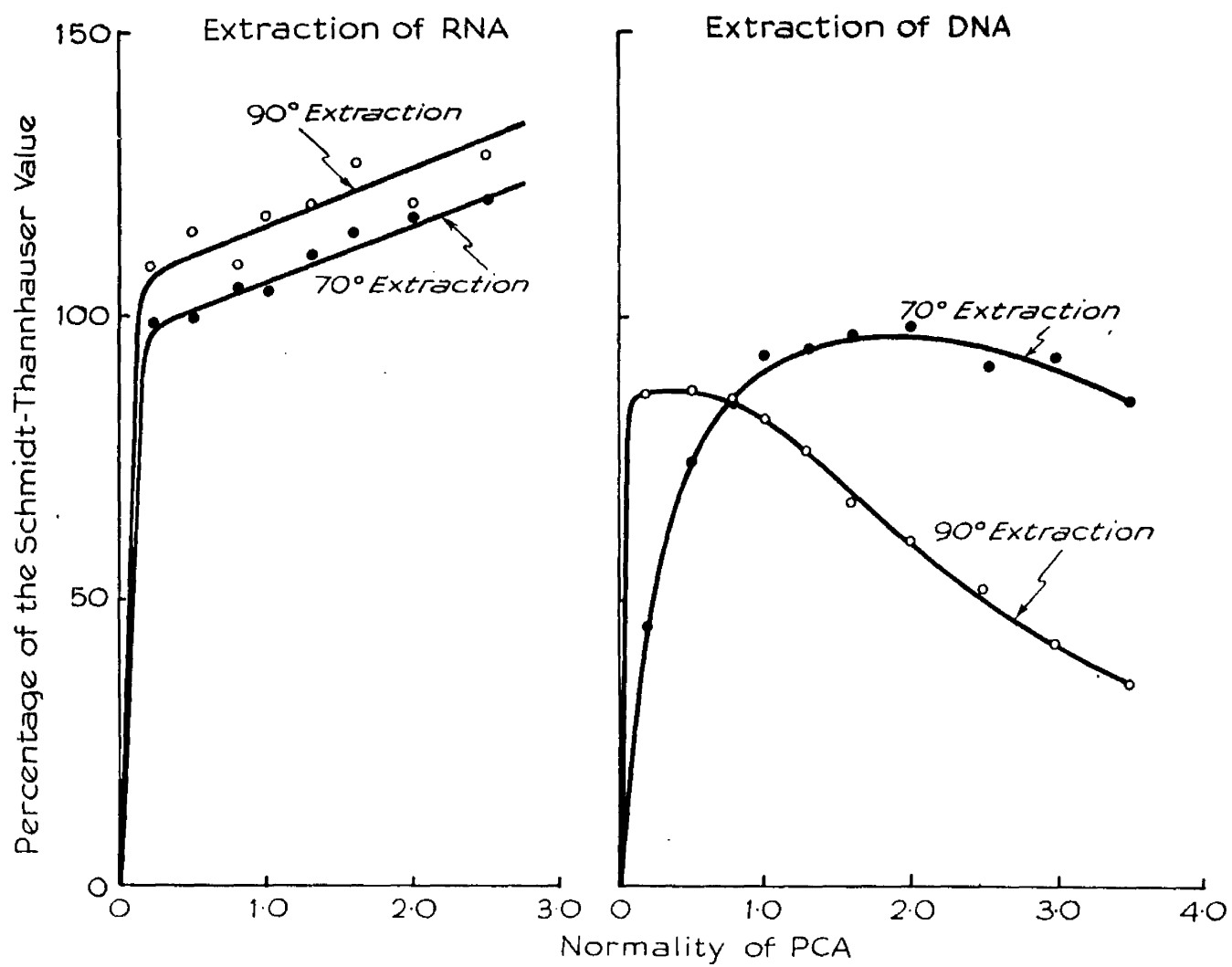


FIGURE 39

procedure for the estimation of DNA. We have therefore preferred to use the alkaline digestion procedure of Schmidt and Thannhauser for nucleic acid estimation.

Thus our final scheme for the analysis of tissue nucleic acid falls into three parts:

- (a) precipitation and washing with cold acid
- (b) extraction with lipid solvents
- (c) analysis of the nucleic acid content of the residue.

The methods found most suitable for the second and third of these steps have already been discussed. We have also carried out an investigation into the efficiency of different acids, and the effect of different times of exposure to the cold acid, from the point of view of nucleic acid precipitation in stage (a). The results of this study are given in Table 54. Samples of liver homogenate were precipitated and washed twice with 0.7N PCA or 10% TCA, the time of contact varying from 0.5 hours (the minimum time needed for these manipulations) up to 24 hours. The samples were then extracted with lipid solvents and analysed for nucleic acids by the Schmidt-Thannhauser procedure. As the table shows, contact with 10% TCA for 24 hours solubilised none of the DNA and only 7% of the RNA, but the samples treated with 0.7N PCA gave lower recoveries of RNA at all times from 2 hours onward. Thus, although neither acid precipitant leads to large losses of nucleic acid, it is clear that, for routine removal of acid-soluble substances, 10% TCA is the safer choice.

Our conclusions from this brief investigation of certain methods of

TABLE 34

The recovery of nucleic acids from rat liver after different periods
of contact with cold TCA or ICA

The total recoveries of RNA and DNA are given as $\mu\text{g.}$ nucleic acid per 100 mg. liver and as percentage change from the values obtained after 0.5 hours' contact with 10% TCA. The results are the mean of those obtained by P estimations and by Ceriotti or orcinol reactions.

Treatment	Period of contact (h)	RNA	DNA
10% TCA	0.5	77.0	22.9
	24	71.2 (-7%)	22.1 (-3%)
0.7N ICA	0.5	74.9 (-3%)	22.9 (+0%)
	2	69.3 (-10%)	22.7 (-1%)
	6	66.2 (-14%)	21.3 (-7%)
	24	62.9 (-18%)	21.0 (-8%)

analysing nucleic acids in liver may be summarised as follows:

(a) Either 0.7N FCA or 10% TCA may be used as a protein precipitant and solvent for acid-soluble material without serious loss of nucleic acids. Contact with FCA for 2 hours or longer, however, causes lower recoveries of RNA, although DNA is unaffected. Thus TCA is probably a safer choice.

(b) RNA is efficiently extracted by two twenty-minute extractions with 0.5N FCA at 70°C, but higher temperatures or concentrations of acid may extract other orcinol-reacting material, and give values which are too high.

(c) The most favourable conditions for extraction of DNA by FCA are (a) a temperature of 70°C and (b) a FCA concentration between 1N and 3N. Even so, the extract contains less than 90% of the DNA of the specimen, as measured by the Schmidt-Thannhauser procedure. The latter method is therefore to be preferred for DNA estimation.

(d) The values obtained by the Cериotti and orcinol reactions for nucleic acid P in fractions prepared by the Schmidt-Thannhauser method (see Figure 9) agree well with their total P content, if allowance is made for the "concomitant" P of the supernatant (acid-soluble) fraction.

(e) The most suitable scheme for routine analysis thus seems to be:

1. precipitation and two washes with 10% TCA
2. extraction with lipid solvents as in Figure 6
3. alkaline digestion and fractionation by the Schmidt-thannhauser method, and measurement of RNA and DNA in the fractions (by P estimations and by specific colour reactions).

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THE PROTEIN COMPONENTS OF SUBCELLULAR STRUCTURES INVOLVED IN PROTEIN SYNTHESIS

by

Eileen D. Downie

SUMMARY:

A nitrogen-containing material which is removed by lipid solvents from liver tissue residues has been investigated chemically and biologically. Certain organic solvents commonly used to extract lipids from trichloroacetic acid-treated residues of liver solubilise more than 20% of the total acid-insoluble nitrogen. Only a small part of this nitrogen is contributed by phospholipid, and little if any by nucleic acid. Results obtained by means of amino-nitrogen and peptide-bound nitrogen estimations, and of paper chromatography, indicate that the nitrogenous material of the solvent extract consists mainly of protein or polypeptide. This "lipid protein" can be separated from the phosphorus-containing substances of the solvent extract by washing with 0.2 volumes of water. The "lipid protein" occurs in all the cell fractions examined (mitochondria, microsomes and cell sap), but is especially abundant in the mitochondria and larger microsomes, fractions which are rich in cell membranes. The distribution of the "lipid protein" follows that of the phospholipid, and it is probably a component of the cytoplasmic membranes.

Chemical treatment of microsomal pellets prepared by differential centrifugation in sucrose solution confirms the suggestion that the "lipid protein" is of membranous origin. The microsomal fraction corresponds to the endoplasmic reticulum of the intact cell, and fractionation of the microsomes by means of a variety of reagents indicates that the "lipid protein" of microsomes is derived from their membranous component rather than the ribosomal granules.

Protein deficiency for 11 days decreases the amount of "lipid protein" in the liver cell, but the other constituents estimated (non-lipid protein, RNA and phospholipid) also fall by the same proportion. Thus protein deficiency appears to lead to the loss of cell structures as whole units rather than the specific loss of any one chemical constituent. The regrowth of endoplasmic reticulum membranes which follows the feeding of a protein meal to fasted rats is accompanied by an increase in the amount of "lipid protein", which thus seems to vary in parallel with changes in the amount of membranous material in the cell.

The lipid solvent extract of the acid-insoluble residue of liver from rats injected 5-60 minutes previously with ^{14}C -leucine contains considerable amounts of radioactivity, which are probably not due to contamination with free leucine. The uptake in the solvent extract, relative to that in the non-lipid protein, is stimulated by the feeding of a protein

meal to rats fasted overnight. This effect is not seen in rats which have been depleted of protein for 6 days; the endoplasmic reticulum may be modified by protein depletion so that it is no longer able to respond to the protein meal. The response to the meal observed in the adequately nourished rats is seen in all the cytoplasmic fractions. The uptake is highest in the microsomes, and especially in the lipid solvent extract of their membranous component. The activity reaches a peak 20-30 minutes after the injection of the radioactive leucine.

The evidence therefore suggests that certain organic solvents are capable of removing from the trichloroacetic acid-treated residue of liver a specific nitrogenous material which is free from phosphorus and is probably protein or polypeptide in nature. This "lipid protein" is probably derived from the membranes of the endoplasmic reticulum, is specifically sensitive to starvation and to re-feeding with protein, and is associated with a highly radioactive fraction in the microsomal membrane 5-30 minutes after the intravenous injection of ^{14}C -leucine.